A role for cell-matrix interactions in the regulation of human bone cell function

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A ROLE FOR CELL-MATRIX INTERACTIONS IN THE REGULATION OF HUMAN BONE CELL FUNCTION

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ABBREVIATIONS

1,25D	-	1,25-dihydroxyvitamin D_3
ATP	-	Adenosine triphosphate
APAAP	-	Alkaline phosphatase anti-alkaline phosphatase
BMP	-	Bone morphogenetic protein
BSA	-	Bovine serum albumin
BSP	-	Bone sialoprotein
cAMP	-	Cyclic adenosine monophosphate
CS-FCS	-	Charcoal stripped foetal calf serum
CFU-F	-	Colony forming unit, fibroblast
CFU-GM	-	Colony forming unit, granulocyte-macrophage
CFU-M	-	Colony forming unit, macrophage
DABCO	-	Diazo-bi-cyclo-octane
DNA	-	Deoxyribonucleic acid
ECM	-	Extracellular matrix
EHS	-	Engelbreth-Holmes-Swarm tumour
FACS	-	Fluorescence activated cell sorter
FCS	-	Foetal calf serum
FITC	-	Fluorescein isothiocyanate
FSC	-	Forward scatter
GM-CSF	-	Granulocyte-macrophage colony stimulating factor
IL-1β	-	Interleukin 1 beta
IGF-I and II	-	Insulin like growth factors I and II
IL-6	-	Interleukin 6
IF	-	Immunofluorescence
IFNγ	-	Interferon gamma
LIF	-	Leukaemia inhibitory factor
mAb	-	Monoclonal antibody
MAX	-	Maximum
M-CSF	-	Macrophage colony stimulating factor
MEM	-	Minimal Essential medium
MFI	-	Mean fluorescence intensity
NRS	-	Normal rabbit serum
NSB	-	Non specific binding
OAF	-	Osteoclast activating factor
OIF	-	Osteoinductive factor
PBS	-	Phosphate buffered saline

PG	-	Prostaglandin
PTH	-	Parathyroid hormone
RIA	-	Radioimmunoassay
SSC	-	Side scatter
TBS	-	TRIS buffered saline
TC	-	Total counts
TGFβ	-	Transforming growth factor beta
TNF	-	Tumour necrosis factor
TRAP	-	Tartrate resistant acid phosphatase

SUMMARY

The underlying mechanisms controlling the complicated process of bone remodelling are complex and poorly understood. One of the many potentially important interactions is that between the cell and its extracellular matrix, various components of which have been shown to exert a number of effects in non bone cell types. Apart from these direct effects, the extracellular matrix is also concerned with the presentation of cytokines and growth factors to neighbouring cells.

Cellular interactions with the extracellular matrix are mediated by specific families of receptor, one of the most well studied of which are the integrins. By binding to different ligands, the cell surface expression of integrin subunits determines the microenvironment immediately surrounding the cell. Through specific cytoskeletal associations, integrin subunits potentially determine the way in which a cell responds to its external environment. In addition to this fairly passive role, the activities of many integrin molecules can be radically modulated by cells, and they in turn, can modulate cell activities in ways that extend far beyond adhesion.

The aim of this Ph.D was to examine the cell surface expression of integrin subunits by human bone cells and to assess their regulation by cytokines and extracellular matrix components. Any modulation of integrin subunit expression was compared with other phenotypic effects elicited by these agents and the significance of any correlations was assessed.

Osteoblasts and osteoclasts expressed different integrin subunits which potentially adhere to different ligands within the bone matrix. Osteoblasts primarily expressed α1, α3 and β1 subunits whilst osteoclasts expressed α2, β1, αV and β3. Staining patterns observed *in situ* for cells of the osteoblastic lineage were maintained in culture but there was increased expression of α2, and αV subunits were weakly positive.

Expression of the major integrin subunits detected on cultured human osteoblast-like cells was regulated by interleukin 1 beta (IL-1β), transforming growth factor beta (TGFβ) and to a lesser extent by 1,25-dihydroxyvitamin D_3 (1,25D). However, changes in expression of α1, α2, α3 and β1 subunits could not be correlated with proliferation, alkaline phosphatase activity or 1,25D induced osteocalcin release. IL-1β and TGFβ also modulated the expression of these subunits in MG-63 cells. Attachment assays were developed using this osteosarcoma cell line but treatment of MG-63 cells with IL-1β and TGFβ did not affect cell adhesion.

Growth of human osteoblast-like cells on fibronectin, laminin, collagen I film and collagen I gel affected cell proliferation, alkaline phosphatase activity and 1,25D induced osteocalcin release. These changes in differentiated osteoblast phenotype could not be correlated with integrin subunit expression. However, when cultured on a collagen I gel, osteoblast-like cells exhibited a different morphology which was accompanied by gel contraction and a 3.5 fold increase in expression of $\alpha 2$ subunits. This data suggests that signals transmitted though the type and level of integrin subunits expressed can influence phenotypic behaviour and hence bone remodelling.

CHAPTER 1.

INTRODUCTION

1.1 THE ULTRASTRUCTURE OF BONE

(reviewed by Baron, 1990 and Teitelbaum, 1990)

The characteristics and structure of bone

Bone and cartilage are specialised connective tissues which together make up the skeletal system. Bone is a highly vascular, mineralised connective tissue which provides structural support and protects the vital organs and bone marrow. Cartilage covers the extremities of bones and provides a smooth articular surface permitting frictionless movement of the joint. In addition to its mechanical and protective roles, bone also serves as a reservoir for ions such as calcium and magnesium in mineral homeostasis.

Bone can be divided into three types. Cortical (compact) and trabecular (spongy) bone together make up lamellar bone and are the major types found in the adult skeleton. Woven bone is primarily associated with the foetus.

Cortical bone

Cortical bone makes up about 80% of the total skeleton and is composed of Haversian systems. An Haversian system (Fig 1.1) consists of plates of bone called lamellae, arranged around a central Haversian canal which contains blood vessels, nerves and lymphatics. Between the lamellae are spaces known as lacunae which contain bone cells (osteocytes) and lymph: the cells communicate via fine channels termed canaliculi which run between the lacunae. Between the Haversian systems there are tiny circular plates of bone called interstitial lamellae (Armstrong and Jackson, 1972).

Cortical bone is found on all external surfaces of all bones. It is thickest in the shaft (diaphysis) of long bones where it encloses the medullary cavity which contains the haemopoietic bone marrow. The functions of cortical bone are mainly structural and protective, its relatively slow turnover suggesting that it does not play a role in mineral homeostasis.

Trabecular bone

Trabecular bone makes up about 20% of the total skeleton and has less well defined Haversian systems. It consists of trabeculae surrounded by stromal spaces containing haemopoietic bone marrow. The surfaces of all trabeculae are generally covered by

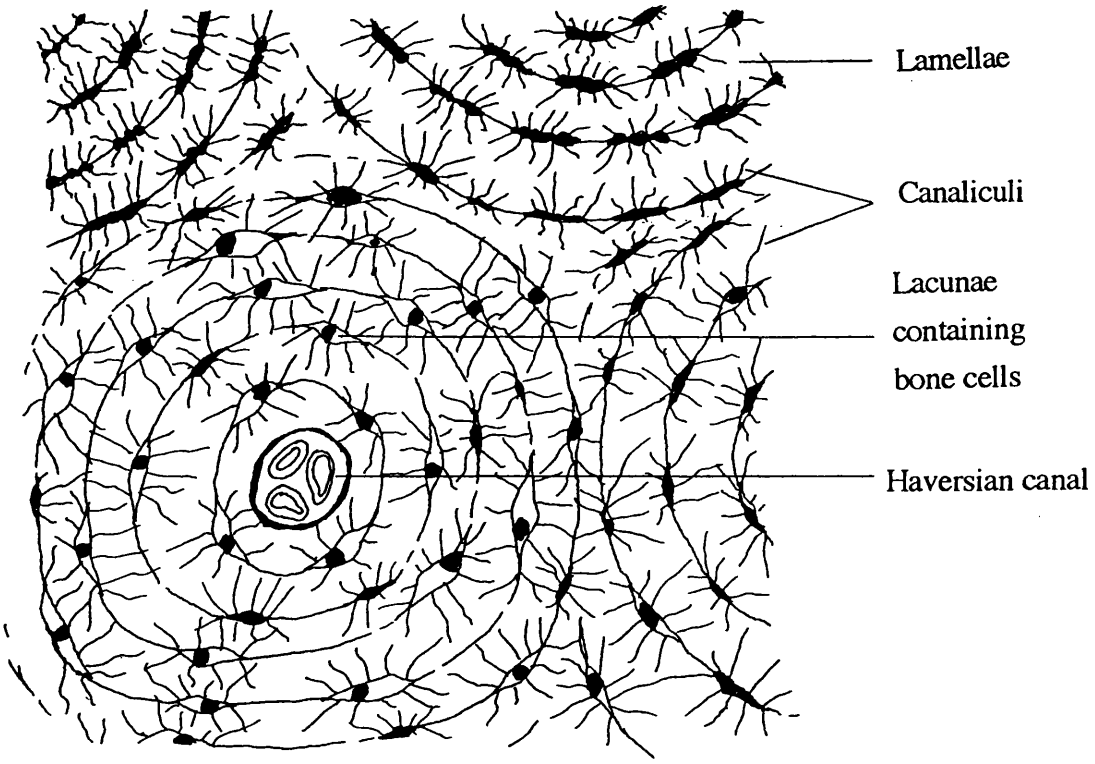
three different types of bone cells. These include the lining cells which are found in metabolically quiescent areas, the osteoblasts in areas of active bone formation and the osteoclasts in areas of active bone resorption.

Trabecular bone is typically found at the ends of long bones and in the vertebral bodies. It is more cellular and vascular than cortical bone and is more metabolically active. Because of its higher rate of turnover, the trabecular bone plays an important role in mineral homeostasis.

Woven bone

Woven bone is the first type of bone to appear in the developing foetus. Here the collagen fibres are orientated randomly as irregular bundles and calcification occurs in disorganised patches. This is an immature form of bone and is mechanically inefficient as a structural unit. It is normally resorbed and replaced by the more organised lamellar bone during development. Woven bone may also appear during fracture healing and in certain pathological conditions such as metabolic bone diseases and tumours.

Figure 1.1 Diagram of an Haversian system (Armstrong and Jackson, 1972)



Bone formation

There are two distinct types of bones found in the skeleton, flat bones and long bones. Flat bones which include the skull, scapula, mandible and ileum are derived from intramembranous bone formation. Long bones which include the tibia, fibula and humerus are derived from both endochondral and intramembranous bone formation. The main difference between these is the presence (endochondral) or absence (intramembranous) of a cartilage phase.

Intramembranous ossification

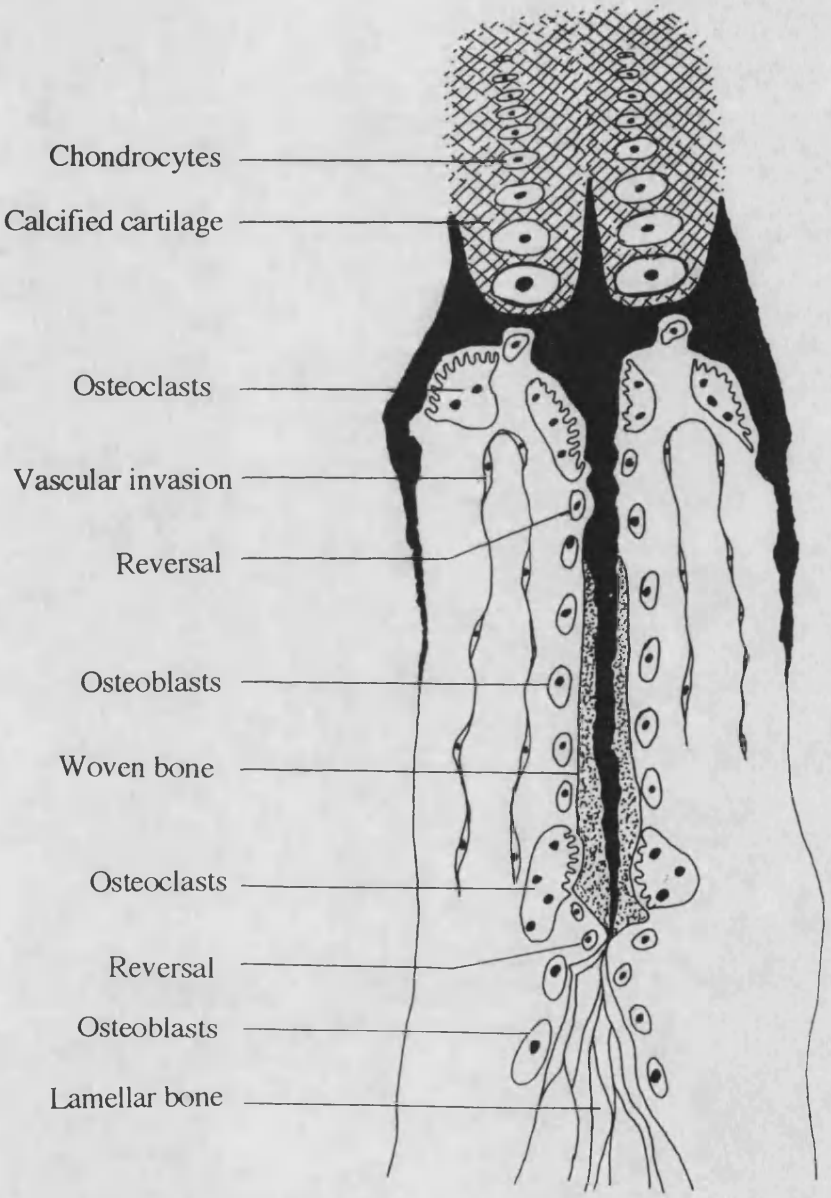
Intramembranous bone formation is characterised by the growth of bone from a cluster of ossification centres in the absence of a cartilage template. At these centres, groups of mesenchymal cells differentiate directly into preosteoblasts and then osteoblasts. These osteoblasts secrete a matrix of type I collagen, proteoglycans and glycoproteins and this subsequently becomes mineralised. This initial trabeculum, lined by osteoblasts, branches into other trabeculae by apposition. As these widen, osteoblasts are incorporated into the matrix and become osteocytes. The early woven bone is resorbed by osteoclasts and later replaced by mature lamellar bone. Those trabeculae destined to form cortical bone continue to expand, incorporating blood vessels into Haversian systems.

Endochondral ossification

In endochondral bone formation (Fig. 1.2), mesenchymal cells proliferate and differentiate into chondroblasts which sit in lacunae and secrete large amounts of cartilaginous matrix. As synthesis continues, cells become embedded in the matrix and form chondrocytes. At the external surface (perichondrium), mesenchymal cells continue to proliferate and differentiate and this allows cartilage to expand by apposition. Chondrocytes embedded in matrix continue to divide and interstitial growth takes place as a result of new matrix synthesis. In the growth plate, chondrocytes appear in regular columns known as isogenous groups. These proliferate and expand the plate interstitially until they enter the hypertrophic zone. Here, hypertrophic chondrocytes enlarge, polarise and produce alkaline phosphatase which is thought to promote subsequent cartilage mineralisation. When formed, the calcified cartilage is partially resorbed by osteoclasts and invaded by blood vessels. A layer of woven bone

is then formed on top of the remaining calcified cartilage. This is remodelled at a later stage to produce mature lamellar bone.

Figure 1.2 Endochondral bone formation (Baron, 1990)



Structure of long bones

A typical long bone consists of a central shaft (diaphysis), an intermediate area (metaphysis) and two ends (epiphyses). The diaphysis consists of dense cortical bone and encloses the medullary cavity which is filled with haemopoietic bone marrow. The epiphyses contain a thinner outer layer of cortical bone and an internal cavity which is filled with bone marrow. There are two surfaces where the bone is in contact with soft tissues; an external (periosteal) surface and an internal endosteal surface. Both these surfaces are lined with osteogenic cells organised in layers to form the periosteum and endosteum respectively. During growth, the metaphysis and epiphysis are separated by a layer of epiphyseal cartilage which makes up the growth plate.

1.2 STRUCTURE OF THE BONE MATRIX

Most of the structure of bone consists of extracellular matrix which has both organic and inorganic components. The organic component is largely responsible for the tensile strength of bone whilst the inorganic component contributes to its compressive strength and rigidity. The organic matrix is composed chiefly of type I collagen but there are also many noncollagenous proteins which may function as instigators and/or regulators of bone remodelling and mineralisation: the properties of some of these will be described below. The inorganic component consists mainly of crystals of hydroxyapatite [$Ca_{10}(PO_4)_6(OH_2)$] which are organised in the same direction as the collagen fibres.

1.2.1 Collagen

Collagen is the most abundant component of the bone matrix, comprising 85-90% of total bone protein. It consists of three polypeptide α chains characterised by unique repeating sequences of the three amino acids Gly-X-Y. X and Y are often proline or its derivative hydroxyproline and it is this sequence which allows the polypeptide chains to form an extremely stable, semi-rigid triple helix. More than ten types of collagen have now been identified, but to date, the collagen found in bone is almost exclusively type I. The type I collagen molecule consists of two identical α1(I) chains and one α2(I) chain wound together in a triple helix to form a collagen fibril. The three constituent α chains of collagen I are synthesised simultaneously and assembled into a helical structure intracellularly. In the matrix, collagen molecules associate head to tail longitudinally and laterally in a one quarter molecule-staggered array. This collagenous

scaffolding then becomes stabilised by the formation of covalent cross links (Gehron Robey, 1989)

1.2.2 Noncollagenous proteins

Osteocalcin

Osteocalcin (bone Gla protein) was the first of the noncollagenous bone matrix proteins to be identified and was discovered by two groups almost simultaneously (Hauschka et al.1975; Price et al.1976). It has a molecular weight of 5.3 Kd, contains one intramolecular disulphide bond and is characterised by three to five residues of γ-carboxyglutamic acid (Gla). This modified amino acid is dependent on vitamin K for its synthesis and has been implicated in calcium binding. Northern blot analysis of extracted mRNA and immunocytochemical studies demonstrate that osteocalcin is mineralised-tissue specific and occurs in young osteoblasts and odontoblasts (Bronckers et al.1985; Bronckers et al.1987). Several osteosarcoma cell lines and non-transformed cultures of osteoblast-like cells synthesise osteocalcin and its production is transcriptionally regulated by 1,25-dihydroxyvitamin D_3 (Price and Baukol, 1980; Beresford et al.1984b). The function of this protein is unknown but it is a potent inhibitor of hydroxyapatite crystal growth *in vitro* (Romberg et al.1986). Furthermore, rats treated with the vitamin K antagonist warfarin, exhibit low bone levels of osteocalcin (less than 2 % of normal) and display an excessive mineralisation disorder characterised by a loss of longitudinal growth and a densely calcified growth plate (Price et al.1982). Taken together these findings suggest that osteocalcin serves as a regulator of mineralisation *in vivo* by inhibiting crystal growth. However, osteocalcin has been shown to exhibit chemotactic activity for two osteoblastic rat osteosarcoma cell lines (Lucas et al.1988) which implies that it may also be important for bone cell recruitment during the process of bone remodelling. A small fraction of osteocalcin is present in the circulation and measurement of serum levels are used as an index of bone turnover.

Osteonectin

Osteonectin is an abundant noncollagenous protein in bone. It is a 32 Kd phosphoprotein which has numerous disulphide bonds and can interact with both collagen and hydroxyapatite (Marks and Popoff, 1988). Osteonectin is not bone specific but levels are typically 1000-10,000 times higher than in other tissues (Gehron Robey, 1989). Immunohistochemical studies have localised this protein in

preosteoblasts, osteoblasts, newly formed osteoid, mineralised matrix and osteocytes (Jundt et al.1987; Bianco et al.1988). It is particularly abundant in developing bone and tends to be found in cells and tissues undergoing rapid proliferation and remodelling. A structurally and immunologically distinct form of osteonectin has also been observed in the α granules of platelets (Malaval et al.1991): its function is unclear but Clezardin et al.(1991) have postulated a role in the maintenance of platelet aggregation. The function of osteonectin in bone is also unknown but its strong binding to hydroxyapatite indicates a possible regulatory function in hydroxapatite crystal growth (Sodek et al.1986).

Osteopontin

Osteopontin is an acidic phosphorylated glycoprotein (44 Kd) which is rich in cysteine residues and contains several disulphide bonds. It binds tightly to hydroxyapatite and contains the functional attachment sequence Arg-Gly-Asp (RGD) (Oldberg et al.1986). Osteopontin has been localised in the extracellular matrix of woven bone and has been found in preosteoblasts, osteoblasts, osteocytes and osteoclasts (Mark et al.1987; Mark et al.1988). More recently, osteopontin mRNA has been detected in osteoclasts (Merry et al.1992; Tezuka et al.1992), indicating that this protein is synthesised by osteoclasts and not purely absorbed from the matrix. This protein is not unique to bone but has also been demonstrated in other tissues including kidney, brain and ear (Nomura et al.1992). Osteopontin is a major secretory product of the rat osteosarcoma cell line ROS 17/2.8. Synthesis is increased by 1,25-dihydroxyvitamin D_3 (Prince and Butler, 1987) and transforming growth factor β (Noda et al.1988) and decreased by parathyroid hormone and other agents which increase intracellular levels of cAMP (Noda and Rodan, 1989).

The function of osteopontin is unknown but the presence of the peptide Arg-Gly-Asp (RGD) suggests that it may play a role in adhesion. In support of this, high levels of osteopontin have been demonstrated in the bone matrix underlying resorbing osteoclasts in the rat, as demonstrated by light microscopy (Mark et al.1987) and immunoelectronmicroscopy (Reinholt et al.1990) (see Chapter 4 discussion).

Thrombospondin

Thrombospondin is a trimeric glycoprotein composed of identical subunits (150 Kd) which are linked by disulphide bonds. The molecule has an RGD sequence (Lawler and Hynes, 1986) which suggests that it is capable of promoting cell attachment but it

was initially found in platelet α granules where it specifically interacts with osteonectin (Clezardin et al.1988). It is now known to be a product of many connective tissue cells and in general is present during stages of proliferation, migration and intracellular adhesion (O'Shea and Dixit, 1988). Thrombospondin is synthesised and secreted by osteoblasts (Clezardin et al.1989; Gehron Robey et al.1989) and osteosarcoma cells (Clezardin et al.1989) and *in vitro* studies have demonstrated that it mediates cell attachment but not spreading (Clezardin et al.1989; Gehron Robey et al.1989). However this effect is dependent on cell type as primary human osteoblast-like cells adhered well but attachment of two osteosarcoma cell lines was inhibited (Clezardin et al.1989). The physiological significance of the RGD sequence is therefore not yet clear. The proposed functions for this molecule range from mediating platelet aggregation, organising extracellular matrix components and acting as an autocrine growth factor (Gehron Robey, 1989).

Bone sialoprotein

Bone sialoprotein (BSP) is an acidic glycoprotein (70-80 Kd) containing no cysteine residues or disulphide bonds (Fisher et al.1990); expression is not regulated by 1,25 - dihydroxyvitamin D_3 (Gehron Robey and Fisher, 1987). *In situ* hybridisation and immunocytochemistry has localised BSP in osteoblasts, osteocytes and in some hypertrophic cartilage of developing long bones and calvariae (Bianco et al.1991). Northern blot analysis originally showed that BSP was fairly bone specific (Fisher et al.1990) but it has since been localised in placenta (Bianco et al.1991). Like osteopontin, there is an RGD attachment sequence which possibly binds to the vitronectin receptor (Oldberg et al.1988; Fisher et al.1990). However, osteopontin is active at lower concentrations and attachment is maintained for longer periods of time in the absence of protein synthesis (Somerman et al.1988).

Fibronectin

Fibronectin consists of a dimer composed of two similar but not identical subunits (each of which is 250 Kd) held together by disulphide bonds. Each subunit contains at least nine functional domains each of which is composed of three types of homologous repeat: the third type of repeat contains the cell attachment sequence Gly-Arg-Gly-Asp-Ser (GRGDS). The role of fibronectin in bone metabolism has not been extensively investigated. However, it is synthesised during bone development and is present around osteoblasts during osteogenesis (Weiss and Reddi, 1980; Weiss and Reddi, 1981). *In vitro*, osteoblasts can use fibronectin as an attachment protein which

suggests that it may play a similar role *in vivo* especially as binding to collagen is sometimes mediated through fibronectin (Kleinman et al.1981). It has also been shown that osteoblasts synthesize fibronectin in culture and that synthesis is stimulated by transforming growth factor β (Wrana et al.1988).

Proteoglycans

Proteoglycans are large molecules that form the ground substance of connective tissues and bone. There are 2 major classes of proteoglycans present in developing subperiosteal bone and these constitute 10% of the noncollagenous bone matrix proteins. Both molecules are composed of a protein core to which there is covalent attachment of long chains of repeating disaccharide units, termed glycosaminoglycans. The large proteoglycan (600 Kd) is removed prior to mineralisation and the small proteoglycan, like all bone matrix proteins, becomes degraded with age. The smaller molecular weight proteoglycan consists of two products, PG-1 (350 Kd) and PG-2 (200 Kd), named biglycan and decorin respectively. Proteoglycans are synthesised by human osteoblast-like cells *in vitro* (Beresford et al.1987) but are not unique to bone. They have also been detected in articular cartilage (Rosenberg et al.1985), nasal cartilage, skin, tendon, sclera, cornea and aorta (Heinegard et al.1985). The function of proteoglycans in developing and mature bone is unknown but it is thought that they interact with growing collagen fibres to regulate the organisation of the extracellular matrix (Gehron Robey, 1989). However, more recent studies have shown that these molecules also bind to and regulate growth factor activity (see below).

1.2.3 Matrix mineralisation

(reviewed by Gehron Robey, 1989 and Marks and Popoff, 1988)

The process of mineralisation is not understood but it results from the combined influences of cells and the extracellular matrix that they produce. Two mechanisms for bone mineralisation have been described. One of these occurs in calcified cartilage and woven bone and the other in lamellar bone. The key events in mineralisation of cartilage matrix appear to be as follows: During matrix synthesis, hypertrophic chondrocytes undergo apoptosis to produce matrix vesicles containing concentrated enzyme activity. Phospholipids within the plasma membrane of these vesicles sequester calcium and this leads to the appearance of the first mineral crystals and the activation of alkaline phosphatase. Crystallisation proceeds at a rate which seems to depend on the presence of inhibitors e.g. pyrophosphates and acidic noncollagenous proteins. Eventually the vesicle ruptures and the newly formed crystals serve as foci

for the propagation of mineralisation. Noncollagenous proteins may bind to these precursors and regulate the direction of growth and final crystal shape. Mineralisation of adjacent collagen initially occurs within fibrils and subsequently between them, producing crystals orientated parallel to the collagen fibres. In lamellar bone, there are less matrix vesicles which are not localised to mineralising sites. Here, mineralisation is initiated in the hole regions, where newly synthesised phosphoproteins are located. This suggests that phosphoproteins may be sufficient initiators of the mineralisation process. Possible roles of these and other noncollagenous proteins in controlling mineralisation are currently being investigated.

1.3 THE CELLS OF BONE

Lining cells

In the adult, the majority of bone surfaces are covered by a layer of flattened inactive lining cells. The function of these cells is unknown but they may serve as nutritional support for osteocytes via gap junctions between cell processes (Stanka, 1975).

Osteoblasts

Osteoblasts are cuboidal, polar, basophilic cells which line the bone matrix at sites of bone remodelling. They are highly metabolically active and form gap junctions which serve as a communication link between adjoining cells and osteocytes (Jeansonne et al.1979; Doty, 1981). They also express high levels of alkaline phosphatase (Fritsch et al.1985), the function of which is unknown. However, in the genetic disorder hypophosphatasia, this enzyme is deficient and patients develop severe osteomalacia, implying that alkaline phosphatase is essential for mineralisation (Whyte, 1990). The function of osteoblasts is to produce calcified bone matrix. Therefore, they synthesise collagen I, osteocalcin, osteonectin, sialoproteins and proteoglycans (Beresford et al.1984a; Gehron Robey, 1989). They also function in the process of mineralisation although their precise role is not yet understood. In addition to their bone forming activities, osteoblasts are also thought to be concerned with regulating some of the actions of osteoclasts.

Osteocytes

Osteocytes are the end cells of the osteogenic lineage and are derived from osteoblasts which have become enclosed within lacunae in the bone matrix. These cells are much

smaller than osteoblasts and are not as metabolically active; features which become more pronounced deeper within the bone matrix. Osteocytes are linked to each other and to osteoblasts through long cytoplasmic processes which extend through canaliculi permeating the bone matrix (Jeansonne et al.1979; Doty et al, 1981; Nejweide et al.1981). The function of these cells is unknown although the viability of bone depends on their presence. There is some data to suggest that they are involved in transducing signals and responding to stress, since a variety of enzymes and RNA synthesis are increased in bones subjected to a stress regime (Skerry et al.1989).

Osteoclasts

(reviewed by Peck and Woods, 1988 and Vaes, 1988)

Osteoclasts are large, differentiated, multinucleated cells which have the capacity to resorb bone and cartilage. They are not commonly seen on quiescent bone surfaces but are often found at sites of active bone remodelling such as in the metaphysis of growing bones. They have also been observed in some pathological conditions, for example adjacent to collections of tumour cells.

Osteoclasts are giant cells derived from fusion of mononuclear precursor cells. They vary greatly in size, but the average number of nuclei per cell ranges from 10 to 20 (Mundy, 1990). One of the unique ultrastructural characteristics of this cell type is the presence of a ruffled border which has numerous folds and invaginations and allows intimate contact with the bone surface. The ruffled border is surrounded by a clear zone which is rich in actin filaments. This so called 'sealing zone' is closely apposed to the bone surface and mediates attachment of the osteoclast to the bone matrix, sealing off a resorption compartment. The rest of the cytoplasm contains large numbers of lysosomes, mitochondria and golgi complexes. There are also many vesicles, thought to contain apatite crystals and collagen fibres derived from recently resorbed matrix. Osteoclasts express a large number of enzymes including tartrate resistant acid phosphatase (TRAP), acid hydrolase, cysteine proteases and carbonic anhydrase II. TRAP and the hormone receptor for calcitonin are the main markers used to identify this cell type.

Resorption occurs at the ruffled border and is a two step process involving demineralisation followed by degradation of the extracellular matrix. Demineralisation results from an acidic environment created between the ruffled border of the osteoclast and the bone surface. The high concentration of H^+ ions is formed from the action of carbonic anhydrase and is pumped out of the cell by an ATP dependent proton pump.

The acidic environment also facilitates subsequent degradation of the extracellular matrix. This is thought to result mainly from the actions of cysteine proteases such as cathepsins L, B and N.

Bone marrow

There are two types of bone marrow found in the adult skeleton. Red marrow is located in trabecular bone and contains an abundance of erythrocytes and blood vessels. The surrounding tissue of red marrow consists of reticular fibres which support various types of progenitor cell and mature leukocytes, and it is therefore a primary site of haemopoiesis. Yellow marrow is found in the medullary cavity of long bones. Like red marrow, it consists of fat and blood cells, but is not so rich in blood supply or erythrocytes (Armstrong and Jackson, 1972).

The osteoblast lineage

Cells of the osteoblastic lineage are considered to arise from a fibroblast colony-forming cell (CFU-F) isolated from the haemopoietic bone marrow (reviewed by Owen, 1988; Beresford, 1989). CFU-F is capable of proliferating and differentiating into fibroblastic, reticular, adipocytic and osteogenic stromal cell lines depending on the culture conditions. The stages of osteoblastic differentiation, from a committed progenitor to the mature osteoblast have not yet been elucidated owing to a lack of suitable biochemical or cell surface markers.

The osteoclast lineage

Osteoclasts are thought to arise from haemopoietic mononuclear cells in the bone marrow (Roodman et al.1985). These precursor cells circulate in the blood and, at sites destined to be remodelled, they proliferate, differentiate and finally fuse to form multinucleated osteoclasts which subsequently resorb bone. The regulation and mechanisms of osteoclast formation are poorly understood but local factors produced by osteoblasts can stimulate both the proliferation and differentiation of osteoclast progenitors. The cell of origin for the osteoclast within the bone marrow is still under debate and currently there are three theories regarding the osteoclast lineage (reviewed by Nijweide et al.1986; Marks and Popoff, 1988). The weight of evidence to date, suggests that the progenitor is a pluripotent stem cell (CFU-GM) which has the capacity, in response to certain stimuli, to differentiate into a granulocyte, monocyte or osteoclast.

1.4 THE BONE REMODELLING CYCLE

(reviewed by Parfitt, 1988)

Bone remodelling consists of a tightly controlled sequence of events occurring at discrete sites in the skeleton. It involves the co-ordinated action of a range of cell types, many of which have still not been fully identified. These include osteoclasts, osteoblasts, osteocytes and possibly haematopoietic, immune and endothelial cells. Bone remodelling occurs mostly at the endosteal surface and its main functions are to replace effete bone and to maintain mineral homeostasis. The remodelling process has been divided into a number of stages as shown in Figure 1.3.

(i) Quiescence

Quiescent bone is covered by a layer of thin, apparently inactive lining cells thought to be primarily of the osteoblast lineage. However, this cell population is undoubtedly heterogeneous and also contains osteoblast and osteoclast precursors, and cells which help to regulate bone remodelling by transmitting signals between cell types.

(ii) Activation

The first observable event during bone remodelling is an increase in the number of mononuclear precursor cells around the resorption site. These activated mononuclear cells insert long pseudopodia down between the flattened lining cells. It is thought that the lining cells may facilitate this process by digesting the endosteal membrane and retracting to expose the bone surface. On reaching the bone surface the precursor cells undergo proliferation, differentiation and finally fusion to form multi-nucleated osteoclasts.

(iii) Resorption

The newly formed osteoclasts, now attached to the bone surface resorb a defined quantum of bone forming a resorption pit or Howship's lacuna approximately 50 μm deep.

(iv) Reversal

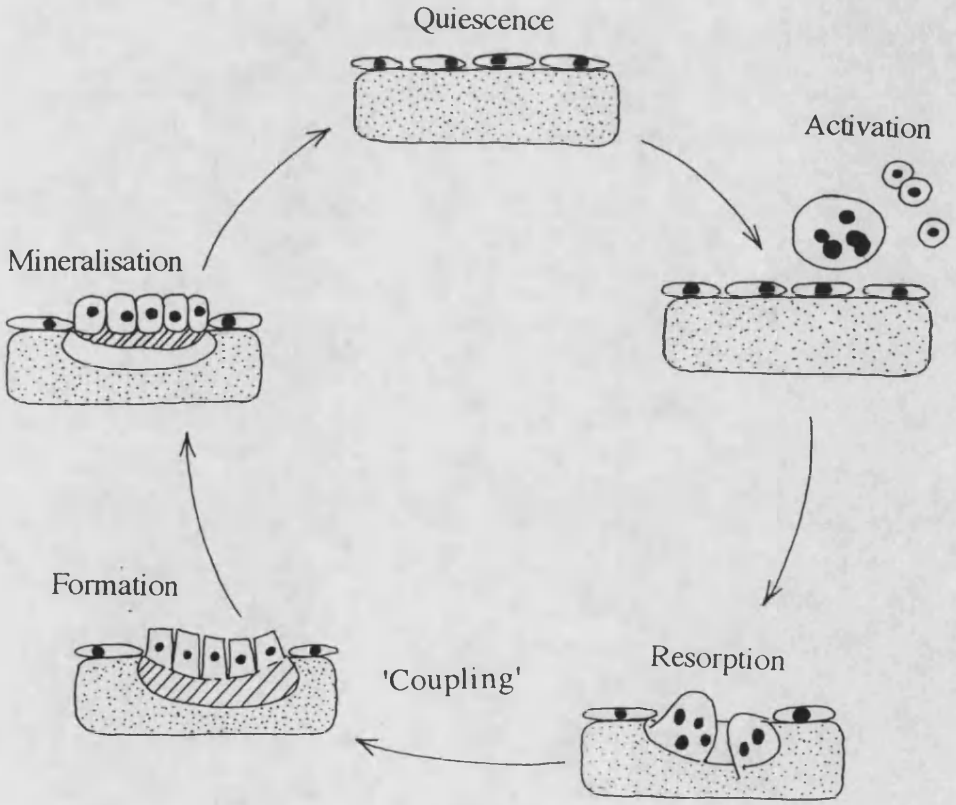
Resorption is followed by a reversal phase, when osteoclasts are replaced by TRAP positive mononuclear cells. The identity of these cells is not known but it has been suggested that they are the products of osteoclast fission. During this phase a cement line is laid down consisting of glycoproteins, glycosaminoglycans and acid phosphatase. This is thought to act as a chemoattractant for osteoblast precursors

which proliferate and differentiate at the resorption site. The release of cytokines into the microenvironment of resorption lacunae and the bone matrix are two other factors which may contribute to the coupling of bone resorption and subsequent formation.

(v) Formation

Newly formed osteoblasts synthesise bone matrix (osteoid) above the cement line. This process continues until the resorption site has been completely replaced. As the osteoid matures it subsequently becomes mineralised.

Figure 1.3 The bone remodelling cycle (Gowen, 1992a)



1.5 SOLUBLE FACTORS AFFECTING BONE REMODELLING

Bone cells are intimately associated with neighbouring cells and extracellular matrix. During the process of bone remodelling, osteoblasts and osteoclasts are regulated by a whole plethora of soluble and insoluble mediators. Systemic hormones, cytokines and prostaglandins are some of the soluble factors which have been shown to affect bone cells and the characteristics and actions of some of these are described below. However, each mediator should not strictly be considered in isolation; it is the interplay between many different local and systemic factors which regulate bone cell metabolism.

1.5.1 Systemic factors

Parathyroid hormone

Parathyroid hormone (PTH) is an 84 amino acid peptide which, after secretion, becomes cleaved between amino acids 33 and 34. The amino terminal fragment retains full biological activity but the carboxy terminal fragment is inactive. The synthesis and secretion of PTH is regulated by serum concentrations of calcium and phosphate and its function is to maintain mineral homeostasis. It is produced in response to decreased calcium concentrations and its major sites of action are in the bone, kidneys and gastrointestinal tract.

In bone, PTH is a potent stimulator of osteoclast mediated bone resorption both *in vivo* and *in vitro*. This effect is achieved by activation of pre-existing osteoclasts (Holtrop and Raisz, 1979; Raisz and Lorenzo, 1980) and by fusion of pre-existing osteoclast precursors (Nijweide et al.1978; Krieger et al.1982; Lorenzo et al.1983). The effects of PTH on bone resorption are indirect (Nijweide et al.1986) as no receptors have been identified on osteoclasts (Silve et al.1982; Rouleau et al.1986) and moreover, PTH does not influence the behaviour of these cells in isolation (Chambers and Dunn, 1982; Chambers et al.1984). It has been speculated that activation of pre-existing osteoclasts occurs via the osteoblast, which expresses PTH receptors (Silve et al.1982; Rouleau et al.1986) and responds both morphogenetically (Jones and Boyde, 1976) and functionally (Kream et al.1980) to PTH *in vitro* . However, it is also possible that osteoclast precursor cells express PTH receptors and that increased cell fusion following PTH administration is a direct effect.

PTH also exerts a number of effects on osteoblasts which can both potentiate bone resorption and affect bone formation. Matrix degradation can be increased by the

release of collagenase (Sakamoto and Sakamoto, 1985) and plasminogen activators (Hamilton et al.1985) which occur in response to PTH administration. In addition, PTH also causes cytoplasmic contraction in osteoblasts, resulting in exposure of underlying matrix for subsequent invasion by osteoclasts (Jones and Boyde, 1976). The effects of this hormone on bone formation are rather more complex. It has been shown to decrease proliferation (Reid et al.1988), collagen synthesis (Dietrich et al.1976; Kream et al.1980) and osteocalcin production (Beresford et al.1984b) but to increase synthesis of prostaglandin E (MacDonald et al.1984). Some of these effects may be mediated through adenylate cyclase or changes in intracellular calcium as concentrations of 3',5'-adenosine monophosphate and Ca^{2+} are increased following addition of PTH (Chase and Aurbach, 1970; Dziak and Stern, 1975).

Calcitonin

Calcitonin is a 32 amino acid peptide secreted by cells from the thyroid in response to increased levels of serum Ca^{2+}. Its main biological effect is to inhibit osteoclastic bone resorption (see Austin and Heath, 1981 for review). However, there are also studies to suggests that it promotes bone formation by stimulating the proliferation, collagen synthesis and alkaline phosphatase activity of osteoblasts (Farley et al.1988; 1991).

Calcitonin receptors have been identified on osteoclasts (Warshawsky et al.1980) and there is every reason to believe that this hormone is a direct-acting inhibitor of osteoclast function. Isolated osteoclasts have been shown to respond to calcitonin within minutes, and pseudopod retraction, cell rounding, reduced motility and cytoskeletal rearrangements are typical morphological changes which occur (Chambers and Magnus, 1982; Chambers and Moore, 1983; Chambers et al.1984). Administration of calcitonin *in vivo* rapidly causes osteoclasts to detach from the bone surface and to lose their ruffled borders (Lucht, 1973). There is also a reduction in the number of osteoclasts and some authors have concluded that calcitonin acts by causing fission of pre-existing osteoclasts into ill-defined mononucleated cells (Baron and Vignery, 1982). Others have detected a change in the localisation of carbonic anhydrase II, and have suggested that this may represent a mechanism of calcitonin-mediated inhibition of bone resorption (Anderson et al.1982). *In vitro*, calcitonin has been shown to transiently inhibit augmented resorption caused by PTH (Feldman et al.1980). This transient effect is reproducible and termed 'escape', but the phenomenon is not understood.

1,25-dihydroxyvitamin D_3

1,25-dihydroxyvitamin D_3 (1,25D) is the major active metabolite of vitamin D and is important in calcium homeostasis. It is produced in response to decreased Ca^{2+} concentrations and its primary sites of action are in the gut, the kidney and bone.

The most striking effect of 1,25D on bone *in vivo* and *in vitro* is the stimulation of osteoclast mediated bone resorption (Raisz et al.1972; Herrmann-Erlee and Gaillard, 1978; Marie and Travers, 1983). This results from an increase in osteoclast number and activity, and is considered to be independent of PTH (Reynolds et al.1976; Holtrop et al.1981). As with PTH, most of the actions of 1,25D on osteoclasts appear to be indirect, implying the involvement of another cell type such as the osteoblast. In support of this, isolated osteoclasts do not respond to 1,25D (Chambers and Dunn, 1982) and receptors have been identified on preosteoblasts and osteoblasts but not on osteoclasts and osteocytes (Narbaitz et al, 1983). It is also possible that 1,25D promotes the differentiation of osteoclast precursors within the bone marrow. This is supported by results obtained from long term cultures of mononuclear cells isolated from feline (Ibbotson et al.1984) and human (MacDonald et al.1987) marrow: in these studies, administration of 1,25D increased the number of multinucleated cells which exhibited several features typical of osteoclasts. Differentiation of other haemopoietic cells is known to occur in response to 1,25D (Bar-Shavit et al.1983; Amento et al.1984; Rigby et al.1985) and this could also stimulate bone resorption through increased secretion of appropriate cytokines.

1,25D also affects mineralisation and bone formation. *In vivo*, it has been shown to promote calcification of bone matrix but inhibit new bone formation, a phenomenon which leads to growth retardation (Reynolds et al.1976; Marie et al.1985). *In vitro* data are often conflicting and the responses of cells seem to depend on variables such as species differences, cell density and stage of differentiation (Rodan and Rodan, 1984). In human trabecular-derived bone cells, 1,25D has been reported to decrease proliferation but to increase collagen I synthesis, alkaline phosphatase activity (Beresford et al 1986) and osteocalcin production (Beresford et al.1984b; Skjodt et al.1985). 1,25D also stimulates collagen I synthesis in the human osteosarcoma cell line MG-63 (Franceschi et al.1988). In ROS 17/2.8 cells, alkaline phosphatase activity (Manolagas et al.1981) and production of osteopontin (Prince and Butler, 1987) are increased, but collagen synthesis is inhibited (Kream et al.1986).

Oestrogen

Oestrogen and androgens stimulate skeletal maturation at puberty and can prevent loss of bone mass in adults. Oestrogen deficiency is clearly central to the development of post menopausal osteoporosis and similarly, androgen deficiency due to the decline of gonadal function in the elderly may be equally important in men.

The mode of action of oestrogen is not understood. Some reports favour an indirect mechanism whereby oestrogen increases serum Ca^{2+} concentrations by enhancing levels of PTH and 1,25D (Riggs et al.1972; Gallagher et al.1980). Others have suggested that oestrogens and other sex steroids affect bone metabolism via the immunomodulatory system (Grossman, 1984). Another possibility is that oestrogen coordinates the production of locally produced cytokines and thereby controls the process of bone remodelling. In support of this, Ernst et al.(1989) demonstrated that oestradiol, in addition to PTH and growth hormone increased the proliferation of rat osteoblasts *in vitro* by increasing insulin-like growth factor I gene transcription. Also, it has been demonstrated that oestrogen decreases bone resorption by inhibiting the production of interleukin-6, a cytokine which is required for haemopoiesis and osteoclastogenesis (Girasole et al.1992; Jilka et al.1992).

There have been numerous studies *in vitro* to investigate any direct effects of oestrogens on osteoblasts. Unfortunately results from these studies appear to be dependent on the cell type and species of origin. For example, in rat cultures, oestradiol has been shown to stimulate osteoblast proliferation in primary cultures (Ernst et al.1988; Ernst et al.1989) but to inhibit proliferation in the osteogenic cell line UMR-106 (Gray et al.1987; Bankson et al.1989). In primary human osteoblast-like cells, treatment with oestrogen appeared to exert no effect (Keeting et al.1991) but in a clonal human osteoblast-like cell line, α1 (I)-procollagen mRNA was increased (Benz et al.1991a). In rat cultures oestrogen also appears to promote a more differentiated osteoblast phenotype as it has been shown to increase expression of alkaline phosphatase (Gray et al.1987), osteocalcin, osteonectin (Glibert et al.1989) and collagen (Ernst et al.1988; 1989).

Despite the well documented effects of oestrogen *in vitro*, localisation of oestrogen receptors in bone has proved difficult. However, specific binding sites have now been detected both in primary human osteoblast-like cells (Eriksen et al.1988) and in rat (ROS 17/2.8) and human osteosarcoma (HOS TE85) cell lines (Komm et al.1988). Primary cultures expressed approximately 1600 binding sites per cell but only 200 sites

were detected in osteosarcoma cell lines. Both values were lower than those observed in cells from the uterus which were shown to express several thousand receptor sites per cell. More recently, oestrogen receptors have also been localised in osteoclasts (Oursler et al.1991; 1992).

1.5.2 Local factors

It is unlikely that systemic hormones alone regulate the tightly controlled and highly localised process of bone remodelling. However, local mediators could provide the necessary site and cell specific signals for recruitment and proliferation of appropriate precursors at the right time. Cytokines and cellular interactions with the extracellular matrix are two local factors which, in combination with systemic hormones, could potentially fulfil these criteria.

1.5.2.1 Cytokines

The first direct evidence that cytokines could affect bone cells was provided by Horton et al. (1972) who demonstrated bone resorbing activity in conditioned medium from human peripheral blood mononuclear cells. This activity was named osteoclast activating factor (OAF) and has since been shown to result from at least 10 different cytokines some of which are described below (see Gowen, 1991 for review).

Interleukin-1

Interleukin-1 (IL-1) exists as two related proteins with virtually identical actions on a wide range of cellular targets. IL-1α and IL-1β are the products of separate genes but are structurally related. Both are translated as a 31 Kd precursor polypeptide which is subsequently cleaved from the C-terminal portion to form 17 Kd mature forms. There is only 26 % amino acid homology and both precursors have no leader sequence suggesting an 'abnormal' route of 'export'. IL-1β is often produced in great excess of IL-1α and is the major secreted form; IL-1α remains cell associated as a membrane bound active form (Gowen, 1992b).

Both IL-1α and IL-1β interact with the same plasma membrane receptor which consists of an 80 Kd glycoprotein. There are approximately 1000 receptor sites per cell and these are of high and low affinity. When IL-1 binds to its receptor, the resulting complex is rapidly internalised (probably by receptor mediated endocytosis) and later dissociated in lysosomes. Once inside the cell, the mechanism of action of IL-1 is

unknown; there is no obvious mechanism of signal transduction but some reports have suggested that it is translocated to the nucleus where it regulates transcription directly (see Martin and Resch, 1988 for review)

IL-1, the first candidate to be identified as an OAF, is a potent stimulator of osteoclastic bone resorption *in vivo* (Boyce et al.1989; Konig et al.1989) and *in vitro* (Gowen et al.1983; Dewhirst et al.1985; Heath et al.1985). IL-1 is active in isolation at 1 pM (Gowen, 1992a) and when present at suboptimal concentrations, acts synergistically with other agents such as tumour necrosis factor α, tumour necrosis factor β (Stashenko et al.1987a) and PTH (Dewhirst et al.1987). This makes IL-1α and IL-1β the most potent, known stimulators of bone resorption *in vitro* (Gowen, 1992a). Cell culture studies suggest that this is achieved by the increased proliferation of osteoclast precursors (Gowen and Mundy, 1986) a finding which correlates with results obtained from long term bone marrow culture experiments (Pfeilschbacher et al.1989). However, there have also been some reports suggesting that the effects of IL-1 are mediated by osteoblasts. For example, Thomson et al.(1986) showed that IL-1 increased bone resorption only when osteoclasts were co-cultured with rat calvarial cells or UMR-106 cells (a rat osteosarcoma cell line): this did not result from the production of prostaglandins, another potent stimulator of bone resorption, as the addition of indomethacin had no effect.

IL-1 also has powerful effects on bone formation. *In vitro*, it stimulates proliferation of human osteoblast-like cells (Gowen et al.1985; Gowen, 1988) and increases DNA synthesis in rat calvarial cells (Canalis, 1986; Smith et al.1987). It also decreases type I collagen synthesis (Beresford et al.1984a; Canalis, 1986) and inhibits alkaline phosphatase activity and 1,25D induced osteocalcin production (Stashenko et al.1987b; Evans et al.1989) suggesting an overall reduction in formation of mineralised bone matrix. In addition, it increases synthesis of prostaglandins and plasminogen activator (Gowen, 1988; Evans et al.1989), two factors which potentially increase the rate of bone resorption.

Most of the literature quoted on the effects of IL-1 on osteoblasts is obtained from studies using normal 'untransformed' bone cell cultures, and it should be noted that findings observed in cell lines are often different. For example, in MG-63 cells (a human osteosarcoma cell line) and in MC3T3-E1 cells (a mouse clonal cell line), IL-1 inhibits proliferation but stimulates alkaline phosphatase activity (Hanazawa et al.1986; Ohmori et al.1988; Dedhar, 1989). This highlights the importance of interpreting *in*

vitro studies with care, and of choosing model systems which are as close as possible to the actual cell of interest.

IL-1 is produced by osteoblast-like cells in culture (Hanazawa et al.1987; Amano et al.1988) and IL-1β mRNA has been detected at specific stages of bone remodelling using *in situ* hybridisation (Dodds et al.1992). The fact that IL-1 is synthesised locally and affects both osteoblasts and osteoclasts suggests that it may play an important role in coupling bone resorption with subsequent formation at individual remodelling sites.

Tumour necrosis factor

Tumour necrosis factor (TNF) exists as a family of two related peptides (α and β) which share approximately 30% homology and are products of separate genes. Like IL-1, TNFα and TNFβ are potent stimulators of bone resorption (Bertolini et al.1985; Tashjian et al.1987). Generally, TNFα elicits a stronger response than TNFβ, but both cytokines are typically between 100-and 1000- fold less potent than IL-1, the active concentration range being 10^{-9}-10^{-7} M (Bertolini et al.1985). The effects of TNFα and TNFβ on bone formation are also similar to those observed with IL-1, although again, higher concentrations of TNF are generally required to elicit a similar response. Thus, TNF (10^{-7}-10^{-10}M) has been shown to stimulate proliferation (Centrella et al.1988; Gowen, 1988; Gowen et al. 1988) and to inhibit collagen synthesis (Canalis, 1987; Smith et al.1987; Centrella et al.1988), alkaline phosphatase activity (Canalis, 1987; Centrella et al.1988) and 1,25D induced osteocalcin production (Gowen et al.1988) in human osteoblast-like cells.

Transforming growth factor β

Transforming growth factor β (TGFβ) is a 25 Kd dimeric peptide which exerts numerous effects in a wide variety of normal and malignant cells. It is synthesised as a larger precursor molecule which is subsequently cleaved and secreted as a biologically inactive form. This latent protein consists of a complex containing the mature dimeric TGFβ together with the 'precursor remainder' and a third component . The active TGFβ dimer can be released by transient acidification, or by the action of exogenous proteases such as plasmin and cathepsin D. Following activation, TGFβ binds to specific cell receptors which are expressed by most cell types (100-10 000 sites/cell). However, the intracellular mechanisms by which it exerts its effects are unknown, since, unlike other growth factor receptors, no associated tyrosine kinase activity has been identified (see Sporn et al.1986; Sporn et al.1987; Massague, 1987 for reviews).

TGFβ exists in three forms, designated TGFβ1, TGFβ2 and TGFβ1.2 (Cheifetz et al.1987). It is present at highest concentrations in platelets but is also very abundant in bone (Sporn et al.1987). TGFβ is particularly prevalent in developing bone which suggests that it may play a role in the regulation of ossification during endochondral bone development (Carrington et al.1988). It is synthesised by bone cells *in vitro* (Gehron Robey et al.1987) and receptors for TGFβ have been identified on primary cultures of human osteoblast-like cells (Gehron Robey et al.1987). Furthermore, its production is increased by the systemic hormones PTH and 1,25D (Pfeilschifter and Mundy, 1987; Merry and Gowen, 1992) so it seems likely that TGFβ could play an important role in the local control of bone cell activity.

In vitro studies suggest that TGFβ modulates the actions of both osteoblasts and osteoclasts. It stimulates cell replication in normal osteoblast-like cells (Centrella et al.1987) but inhibits DNA synthesis in osteosarcoma cell lines (Pfeilschifter et al.1987). TGFβ also stimulates collagen synthesis and alkaline phosphatase activity in some (Centrella et al.1987; Pfeilschifter et al.1987) but not all (Centrella et al.1986) experimental models. The effects of TGFβ on bone resorption are more complex. It stimulates bone resorption in newborn mouse calvarial cultures by a prostaglandin synthesis-dependent mechanism (Tashjian et al.1985). However, in foetal rat long bones, bone resorption is inhibited (Pfeilschifter et al.1988). This inhibitory action of TGFβ may result from decreased osteoclast formation, a finding which is observed when TGFβ is added to bone marrow cultures (Chenu et al.1988).

The mechanism of action of TGFβ in bone is still a matter of controversy. There is some evidence to suggest that the proliferative effects are mediated by proto-oncogenes such as *c-sis*, *c-fos* and *c-myc* (Loef et al.1986; Sandberg et al.1988). Other phenotypic responses may result from a modulation of receptor binding of calcitropic hormones (Merry and Gowen, 1992; Schneider et al.1992), but it has also been suggested that changes in cell-matrix interactions are responsible for the pluripotent effects (Roberts et al.1992).

Insulin-like growth factors

Insulin-like growth factors (IGF-I and IGF-II), have been shown to stimulate the proliferation (Canalis, 1980; Schmid et al.1983) and the production of collagen type I (Canalis, 1980; McCarthy et al.1989; Schmid et al.1989) by osteoblasts *in vitro*. Their biological activities are modulated at least in part by a series of specific high affinity

binding proteins which are synthesised by osteoblasts (Mohan and Baylink, 1991). There is some evidence to suggest that IGF-I mediates the effects of oestrogens. For example Ernst et al.(1989) have shown that IGF-I is produced by bone cells stimulated with oestrogen, and the actions of oestrogen similar to those of IGF-I, can be abated by the addition of anti-IGF-I antibody. Furthermore, the production of IGF binding proteins is also modulated by systemic hormones such as growth hormone (Ernst et al.1990).

Other cytokines

There are a large number of other cytokines active in *in vitro* model systems. Interferon gamma (IFNγ) selectively inhibits cytokine (IL-1, TNF) but not hormone induced bone resorption *in vitro* (Gowen and Mundy, 1986). It also antagonises IL-1 and TNF actions on osteoblasts, in that it inhibits proliferation and stimulates some (alkaline phosphatase and osteocalcin) (Gowen and Mundy, 1986) but not all (collagen) (Smith et al.1987) aspects of differentiated cell function. Bone morphogenetic proteins (BMP's) 1-7 (Wang et al.1988) and osteoinductive factor (OIF) (Bentz et al.1989) are examples of matrix derived cytokines. The BMP's are all members of the TGFβ superfamily but OIF is similar in structure to the bone proteoglycans and therefore has no growth promoting activity of its own. In view of the ability of decorin to bind TGFβ, it has been proposed that OIF may serve as a binding protein for the BMP's and that bone proteoglycans may act as a store for cytokines in bone (Seyedin and Rosen, 1990). Granulocyte-colony stimulating factor (G-CSF) (Felix et al.1988) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Felix et al.1988; Horowitz et al.1989) have been shown to be produced by osteoblasts in culture and are considered to regulate the proliferation and differentiation of haemopoietic precursors. Interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF) are also considered to stimulate osteoclast precursor proliferation (Reid et al.1989; Brandt et al.1990) and overexpression of LIF in transgenic animals leads to elevated bone remodelling and ectopic calcification (Metcalf and Gearing, 1989).

1.5.2.2 Prostaglandins

Prostaglandins (PG's) are a diverse group of unsaturated oxygenated fatty acids whose synthesis is inhibited by indomethacin. They exist in a variety of different tissues and have limited, localised biological activity before being rapidly degraded (Hinman, 1972): the major types localised in bone are PGE_1, PGE_2 and PGI_2.

PG's are potent stimulators of bone resorption (Klein and Raisz, 1970) and some evidence suggests that this results from direct actions on osteoclasts (Chambers and Dunn, 1982; Chambers and Ali, 1983). However, data obtained from studies using mouse foetal long bones (Akatsu et al.1991), suggest that PG's also stimulate osteoclast formation at an early stage of development. In addition to its effects on bone resorption, PGE_2 enhances bone formation by stimulating the replication and differentiation of osteoblast progenitors (Chyun and Raisz, 1984).

1.6 THE EXTRACELLULAR MATRIX

Signals from the extracellular matrix (ECM) can exert as much control over the behaviour of cells as hormones, cytokines and other soluble mediators. The main difference is that the ECM is insoluble and therefore exerts its effects over a short range. Contact with the ECM is fundamental to many biological processes including embryonic development, tissue remodelling, wound healing, inflammation and metastasis (von der Mark et al.1992). Very little information is available concerning the role of of the ECM in regulating bone cell function. However, the process of bone remodelling must be regulated by highly site and cell specific signals which allow recruitment and proliferation of the appropriate cells at the right time. The ECM of bone fulfils all these criteria and as over 90% of bone is composed of matrix, it seems likely that this insoluble local mediator plays an important role in controlling bone cell function.

ECM's are chiefly composed of collagen(s), proteoglycans and glycoproteins. Its major functions are to provide anchorage and support, cues for migration and signals for growth and differentiation. Adhesive and migratory properties of the ECM have been illustrated *in vitro*: if mesenchymal cells are plated onto a surface coated with limiting concentrations of ECM protein applied as a gradient, cells adhere and subsequently move towards the higher concentration (Basara et al.1985). The importance of the ECM in expression of a differentiated cell phenotype can also be observed *in vitro* using chondrocytes. When these cells are cultured on agar or in agarose, they exhibit a rounded morphology and express collagen type II and chondroitin sulphate proteoglycan. If however they are cultured on tissue culture plastic or on glass, they spread out, develop stress fibres and focal contacts, and synthesize type I collagen and non cartilage-type proteoglycans. Under the latter conditions, chondrocytes have become 'fibroblastic' or 'de-differentiated' whereas when cultured on agar or in agarose they express markers characteristic of the differentiated chondrocyte phenotype (reviewed by von der Mark et al.1986).

The importance of the extracellular matrix in expression of a differentiated cell phenotype can also be observed using primary cultures of mouse mammary epithelial cells. When these cells are cultured on plastic or on flat collagen gels, they rapidly lose their ability to synthesize and secrete most milk proteins, even in the presence of lactogenic hormones. If however, they are cultured on 'released' (or floating) type I collagen gels or on a reconstituted basement membrane derived from the Engelbreth-Holmes-Swarm tumour (EHS), there is increased expression of β-casein and transferrin mRNA and enhanced rates of milk protein secretion (Emerman and Pitelka, 1977; Lee et al.1984; 1985; 1987; Li et al.1987). This induction is accompanied by profound morphological changes: cells cultured on plastic or on flat collagen gels display short stubby microvilli on their apical surfaces, they exhibit a flattened morphology and lack internal polarisation and secretory apparatus. In contrast, when cultured on 'released' collagen I gels, cells become columnar and polar and develop tight junctions. Furthermore, extensive microvilli are observed over their apical surface and evidence of a secretory apparatus is displayed internally (Emerman and Pitelka, 1977). Cells grown on EHS are also morphologically differentiated and, in addition, are surrounded by a continual basal lamina separating them from the matrix in which they were embedded (Bissell and Barcellos-Hoff, 1987). These changes in cell structure are accompanied by changes in the organisation of cells into tissue-like forms. The most striking of these occurs following growth on EHS. Here, cells are oriented with their apices inwards and form domes and duct-like structures resembling secretory alveoli (Bissell and Barcellos-Hoff, 1987). This difference in morphology is accompanied by vectoral secretion of milk proteins into the lumen when cells are cultured in the presence of lactogenic hormones (Barcellos-Hoff et al.1987).

1.7 THE INTEGRINS

The integrins are a family of cell surface receptors which mediate interactions between cells and ECM's. Expression of these proteins is essential to many of the functions ascribed to the ECM. For example, integrins play an important role in tissue repair (Clark, 1990), gastrulation (Darribere et al.1988) and tumour cell metastasis (reviewed by Albeda and Buck, 1990). In addition, they are required at several stages of embryogenesis, namely during neural crest migration (Bonner-Fraser, 1985; Bonner-Fraser, 1986), neurite extension (Reichardt et al.1988) and histogenesis (Menko and Boettiger, 1987; Jeffredo et al.1988). Integrin subunit expression is also necessary for the functioning of non adherent cell types and platelets, where it influences the processes of inflammation, immune cell function and blood clotting (Hynes, 1992).

Integrin subunits are expressed by most cell types and defective function causes pathological conditions. Examples include leukocyte deficiency syndrome (Springer et al. 1987), which is characterised by the inability of myeloid cells to carry out phagocytosis and chemotaxis, and Glanzmann's thrombasthenia (Phillips and Agin, 1977) where platelets fail to aggregate in response to activation.

1.7.1 Integrin diversity

The integrins comprise a family of membrane spanning heterodimers composed of non covalently linked α (120-180 Kd) and β (90-110 Kd) subunits (reviewed by Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Albeda and Buck, 1990). 8 β subunits and 14 α subunits have now been identified (Table 1), and most of these have been sequenced at the cDNA level, with the exception of α7 and α IEL (Hynes, 1992). It was originally thought that the α subunits within each group bound faithfully to an individual β subunit, but it can be seen from Table 1.1 that alternative pairings are possible. For example, α4 and α6 can both associate with two β's, and αV can bind up to five different β subunits. When ligand-binding specificities of integrins are examined, two more general features are apparent. First, certain integrins such as α3β1 and αVβ3 are able to bind multiple ligands. Secondly, the same ligand may recognise multiple integrin receptors; for example, both fibronectin and laminin are recognised by six or more different integrin molecules. Yet a further level of complexity is introduced by the existence of alternative splicing which occurs in the cytoplasmic domains of β1, β3, β4, α3 and α6 subunits and in the extracellular domains of αIIb (summarised by Hynes, 1992).

1.7.2 Adhesive ligands and their binding sites

It can be seen from Table 1.1 that the vast majority of integrin molecules bind to ECM proteins and thereby mediate cell-matrix interactions. Other integrins bind to cell membrane proteins ('counter-receptors'), thereby mediating cell-cell adhesion. Considerable progress has been made in defining the integrin recognition sites in the ligands and counter-receptors listed in Table 1.1. The first binding site to be defined was the tripeptide Arg-Gly-Asp (RGD) which is found on many of the ECM components of bone. Examples include collagen, fibronectin, vitronectin, laminin, osteonectin, osteopontin, bone sialoprotein and thrombospondin (reviewed by Humphries, 1990). However, this sequence is not always responsible for mediating adhesion. For instance, α2β1 binds Asp-Gly-Glu-Ala (DGEA) in type I collagen

Table 1.1. The integrin receptor family (Hynes, 1992)

β Subunit	α Subunit	Ligands and counter-receptors
β1	α1	Collagens, laminin
	α2	Collagens, laminin
	α3	Fibronectin, laminin, collagens
	α4	Fibronectin, VCAM-1
	α5	Fibronectin
	α6	Laminin
	α7	Laminin
	α8	?
	αV	Vitronectin, fibronectin, collagen, fibrinogen, von Willebrand factor
β2	αL	ICAM-1, ICAM-2, ICAM-3
	αM	C3b component of complement (inactivated), fibrinogen, factor X, ICAM-1
	αX	Fibrinogen, C3b component of complement (inactivated) ?
β3	αIIb	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin
	αV	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin, collagen
β4	α6	Laminin
β5	αV	Vitronectin
β6	αV	Fibronectin
β7	α4	Fibronectin, VCAM-1
(=βp?)	αIEL	?
β8	αV	?

(Staatz et al.1991) and α4β1 binds Glu-Ile-Leu-Asp-Val (EILDV) in an alternatively spliced region of fibronectin (Humphries et al.1987). Other binding sites have not been identified as precisely although different laminin receptors recognise various parts of the molecule. For example, α3β1, α6β1 and α7β1 bind to the long arm of the laminin molecule, whereas α1β1 and α2β1 recognise the cross region of this ECM protein (reviewed by Hynes, 1992).

Although the presence of cell-binding activity in short, linear peptide sequences is a common feature of ECM molecules, it should be noted that synthetic mimics of these sequences have a relatively low biological activity compared to the molecules from which they are derived. This may result from the three dimensional conformation of the active site peptide or, alternatively, adhesive proteins may contain additional regions that contribute to the affinity and specificity of receptor binding. In support of this, fibronectin, thrombospondin and von Willebrand's factor all possess cell adhesion domains distinct from their RGD sites (Humphries, 1990). In the case of fibronectin, deletion mutagenesis has been employed to identify a site which functions in synergy with RGDS (Obara et al.1988). This site cannot be described by a short amino acid sequence and probably requires precise folding of non contiguous regions of the polypeptide backbone for full biological activity.

1.7.3 Integrin subunit structure

(reviewed by Albeda and Buck, 1990; Hogg, 1991 and Hynes, 1992)

Integrin α and β subunits are unrelated transmembrane glycoproteins with a large extracellular domain, a single hydrophobic transmembrane segment, and a short cytoplasmic domain (50 amino acids or less). A notable exception to this is the cytoplasmic domain of the β4 subunit which comprises over 1000 amino acids. Both subunits are required for ligand binding but only extracellular domains (>75 Kd for β subunit and >100 Kd for α subunit) are required for the association of αβ heterodimers. In support of this, truncated forms lacking transmembrane and cytoplasmic regions can be expressed, and do form αβ heterodimers (Dana et al.1991; Bodary et al.1991). Electron microscopic images of several integrins show a globular head apparently comprising parts of both subunits, and two stalks extending through the lipid bilayer into the cytoplasm. A structural representation of integrin receptors is illustrated in Figure 1.4.

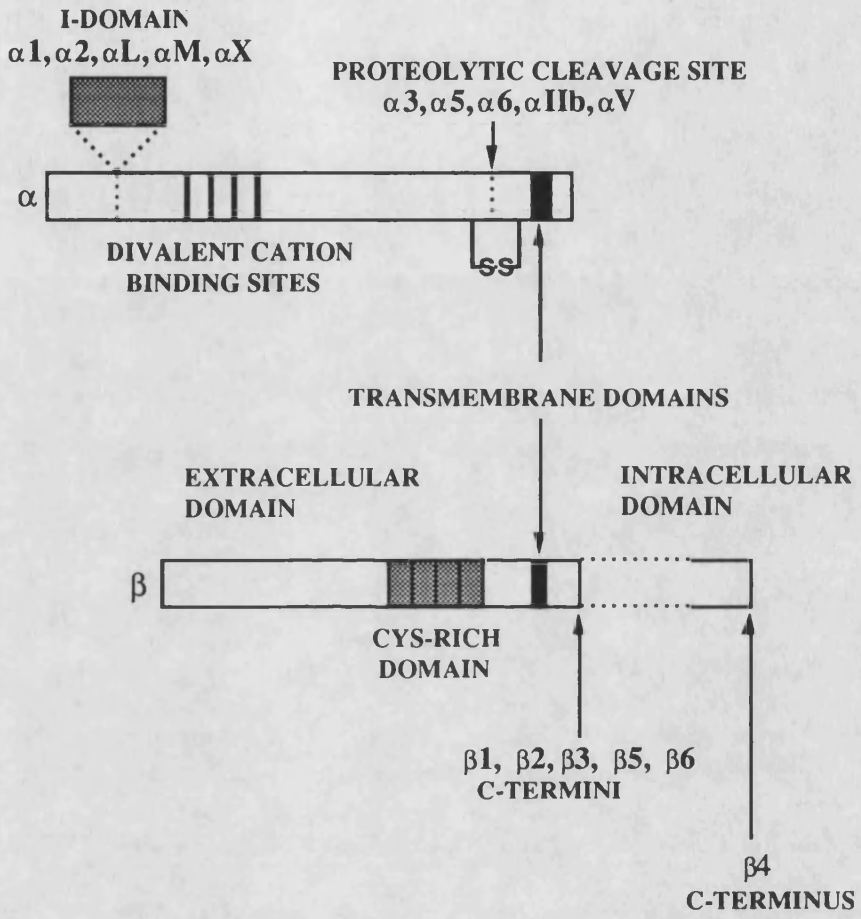
Integrin α subunits are very heterogeneous, and only exhibit an overall 25% amino acid homology which increases to 45-63% between members of the same group. All α

subunits typically possess calmodulin-type divalent cation binding sites close to the ligand binding region in the extracellular domain. In addition, most α subunits contain a proteolytic cleavage site which separates the heavy extracellular chain from the light transmembrane chain, and in some cases these are held together by a disulphide bond. Some α subunits also contain an 180 amino acid insertion, termed the I domain, which is homologous to that observed in collagen binding proteins. The function of this domain is unknown but is considered to contribute to ligand binding. Integrin β subunits share an overall 37-45% amino acid homology, the greatest of which occurs in the cytoplasmic and transmembrane domains. These subunits have an extracellular cysteine-rich region, believed to be disulphide bonded. The amino terminal 40-50 Kd is tightly folded with intrachain disulphide bonds and this associates with the α subunit to form an extracellular ligand binding domain. Divalent cations are essential for receptor function: the nature of the cations can affect both affinity and specificity for receptor-ligand interactions and furthermore, their presence is required for association of some α and β subunits (Gailit and Ruoslahti, 1988; Kirchhofer et al.1990a; Kirchhofer et al.1991).

The cytoplasmic domains of most integrin subunits associate with the cytoskeleton at focal contacts and adhesion plaques. The only known exception to this occurs with the integrin α6β4 which is specifically concentrated at hemidesmosomes in epithelial cells (Stepp et al.1990; Kurpakus et al.1991; Sonnenberg et al.1991) and interacts with microfilaments. The cytoplasmic domains of other integrin molecules interact with the actin cytoskeleton via an indirect linkage involving cytoskeletal proteins such as talin, vinculin and α-actinin and perhaps other cytoplasmic components. There is direct evidence for the involvement of the β1 subunit in mediating cytoskeletal interactions since deletion of all or part of the β1 cytoplasmic domain interferes with associations with focal contacts (Hayashi et al.1990; Marcantonia et al.1990; Solowska et al.1991). The α subunits are also considered to interact with the cytoskeleton although evidence for this association is less direct. It has been suggested that the α subunits convey some kind of functional specificity since the cytoplasmic domains of these subunits are very heterogeneous. However, β1 subunits must also provide some kind of specificity as αVβ1, αVβ3 and αVβ5 do not all associate with the same ligands (see Table 1.1).

There is a tyrosine phosphorylation site on the cytoplasmic domain of the β subunit which suggests that a phosphorylation event may in some way contribute to receptor-cytoskeleton binding.

Figure 1.4 A structural representation of integrin receptors (Humphries, 1990)



1.7.4 Integrin receptor function

Integrin molecules play a structural role in mediating cell adhesion, and in addition, they transmit signals between the external environment and the interior of the cell. The cell surface expression of integrins may be a crucial determinant in the phenotypic behaviour of a given cell type, especially since the ECM with which many integrins interact is known to exert profound effects on cell phenotype. A key feature apparent from Table 1.1 is that there is a tremendous amount of redundancy in the ligand specificity of the integrins. One explanation for this redundancy is that in a given cell, a particular integrin may not exhibit all the specificities listed. For example, α2β1 behaves exclusively as a collagen receptor on platelets (Staatz et al.1989), but on other cell types, it recognises both collagen and laminin (Elices and Hemler, 1989; Kirchhofer et al.1990b). Alternatively, different integrins could transduce specific signals to the interior of the cell, whilst interacting with the same ligand. In support of

this, different αβ heterodimers exhibit different cytoskeletal associations even when interacting with the same ligand. For example, α5β1 is present in focal contacts whereas α3β1 is not, even though both bind to fibronectin (Elices et al.1991). Similarly, αVβ3 is found in focal contacts, whereas αVβ5 is not, even though both interact with the RGD site on vitronectin (Wayner et al.1991). Cytoplasmic domains of various α subunits have also been shown to elicit specific functions. For example, Chan et al.(1992), using chimeric constructs, recently demonstrated that α2 cytoplasmic domains mediated collagen gel contraction whereas α4 cytoplasmic domains promoted cell migration.

1.7.5 Mechanisms of integrin receptor function

Quantitative changes in cell-matrix interactions

Communication between the external environment and the interior of the cell could occur through quantitative changes in the expression of integrin receptor molecules or their respective ligands. It is now becoming apparent that factors known to influence cell phenotype *in vitro* also affect cell-matrix interactions. For example, IL-1β has been shown to modulate expression of various integrin subunits in osteosarcoma cell lines (see Chapter 5). In addition, IL-1α, IL-1β and TNFα have also been shown to modulate collagen deposition by human dermal fibroblasts (Mauviel et al.1991). TGFβ is another cytokine which has been shown to affect cell-matrix interactions. For instance, Roberts et al. (1988) and Heino et al. (1989) have demonstrated an up-regulation of integrin subunit expression in human lung fibroblasts. Similarly, Ignotz and Massague (1987) showed an increased integrin subunit expression using a variety of different cell types, and Ignotz et al. (1989) demonstrated increased expression of vitronectin receptor and LFA-1 following treatment with this cytokine. There have also been several reports of increased matrix deposition following addition of TGFβ. For example, it has been shown to increase fibronectin and collagen mRNA levels in rat (Ignotz et al.1987) and human fibroblasts (Ishikawa et al.1990) and to increase vitronectin expression in Hep G2 hepatoma cells (Koli et al.1991). It has also been found to increase expression of chondroitin/dermatan sulphate proteoglycans in a variety of different cell lines (Bassols and Massague, 1988) but to up-regulate biglycan and down-regulate decorin in human fibroblasts (Kahari et al.1991). Similar studies have also been performed on osteoblast-like cells and a modulation of integrin subunit expression and increased ECM synthesis has typically been observed. For example, Heino and Massague (1989) demonstrated that TGFβ affects the pattern of integrin subunits expressed in MG-63 osteosarcoma cells and causes a selective loss of

adhesion to laminin. Strong et al. (1991) demonstrated increased synthesis of type I collagen in human osteoblast-like cells isolated from explants of trabecular bone, and Noda et al. (1988) showed an increased production of osteopontin in ROS 17/2.8 cells.

Other agents known to modulate bone cell function have also been shown to affect interactions between cells and their external environment. For example, 1,25D increases fibronectin (Franceschi et al.1987) and collagen synthesis (Franceschi et al.1988), and stimulates fibronectin mediated cell adhesion in MG-63 cells (Franceschi et al.1987). 1,25D also increases αVβ3 expression in avian osteoclast precursor cells isolated from marrow (Medhora et al.1993) but glucocorticoids decrease expression of β1 in ROS 17/2.8 cells (McCarthy et al.1992). IGF-II increases collagen synthesis in osteoblast-like cells isolated from explants of trabecular bone (Strong et al.1991) and retinoic acid (10^{-9}M) increases adhesion of the human osteosarcoma cell line OHS-4 to fibronectin (Dunlay et al.1992). In addition, retinoic acid (10^{-5}M) and PMA (10 nM) increase expression of α1 in human endothelial cells, and costimulation with TNFα (5 ng/ml) and PMA result in a synergistic effect (Defilippi et al.1991b). This induction of α1 subunit expression affected the adhesive properties of endothelial cells: attachment to collagen, laminin and laminin fragment P1 was increased, whilst adhesion to fibronectin and laminin fragment E8 remained constant. In another study, TNFα (5 ng/ml) and IFNγ (100 U/ml) decreased expression of β3 which combines with αV to form a vitronectin receptor. This reduction in β3 subunit expression resulted in a decreased adhesion to vitronectin whilst adhesion to fibronectin remained unchanged (Defilippi et al.1991a).

Modulation of integrin receptor affinity and specificity
(reviewed by Hynes, 1992)

Individual cells can vary their adhesive properties by regulating the specificity and affinity of a given integrin molecule. Both activation and de-activation of integrin functions have now been reported but the best understood examples are αIIbβ3 on platelets and β2 integrins on neutrophils, monocytes and lymphocytes. On resting circulating platelets, although αIIbβ3 can attach to surface-bound fibrinogen, it cannot bind to any of its soluble ligands. However, on activation by thrombin, collagen or other platelet agonists, αIIbβ3 undergoes a conformational change and becomes an effective receptor for soluble fibrinogen and the other ligands listed in Table 1.1. The β2 integrins expressed on leukocytes exhibit activation phenomena that are strikingly similar to those exhibited by αIIbβ3: activation of leukocytes is required for expression of the various ligand-binding activities of the β2 integrins (see Table 1.1).

The mechanisms by which these activation events occur are largely unknown. However, evidence from recombinant-DNA expression experiments has suggested that the cytoplasmic domains of integrin subunits are targets for regulatory events.

In addition to these relatively well studied examples of integrin modulation, there are other cases in which intracellular events affect integrin receptor affinity. For instance, activation of T cells by antigen or phorbol esters leads to activation of α2β1, α4β1, α5β1 and α6β1 without changes in surface levels. In contrast to these activation events, there are also examples in which integrins lose activity during development. This occurs with α5β1 during the terminal differentiation of keratinocytes (Adams and Watt, 1990) and with α6β1 during the development of retinal neurons. As with β2 and β3 integrins, the mechanisms by which these regulatory events occur are not understood. However, studies performed by Hotchin and Watt (1992) have demonstrated that loss of α5β1 from the cell surface of keratinocytes involves changes in both transcriptional and post-translational control.

Mechanical transduction via the integrins
(reviewed by Ingber, 1991)

Mechanical forces play a major role in the regulation of tissue formation and function. Cells generate tension within their actin cytoskeleton and exert this force on their extracellular adhesions. At the same time, ECM substrata resist these forces and transmit mechanical forces into the cytoskeleton. This balance between inward and outward forces has been found to be critical for control of cell shape, migration, growth, differentiation and tissue patterning. ECM substrata convey different regulatory signals depending on whether or not they can resist forces from the cytoskeleton. For example, when fibroblasts are cultured on attached collagen gels, they proliferate, spread out, organise long actin bundles and secrete ECM proteins. However, if these gels are 'released', cells become rounded, disrupt their actin fibres, decrease matrix synthesis and become quiescent within 1 hr. In mouse mammary epithelial cells, growth on a 'floating' collagen gel induces expression of differentiation-specific genes and deposition of a basement membrane (Emerman and Pitelka, 1977).

Integrins physically link actin-associated proteins (e.g talin, vinculin, α-actinin, paxillin) to ECM and to adhesion receptors on the surfaces of other cells. Therefore these molecules would potentially alter cell function by affecting the number, location or strength of the adhesive contacts that can transmit physical forces to and from the

cytoskeleton. The importance of integrin molecules in mechanical force transduction can be observed *in vivo* during wound healing. In this process, fibroblasts produce wound contraction and connective tissue remodelling by exerting tension on their ECM adhesions. Control of F-actin bundle assembly, transmembrane force transduction and wound organisation all appear to depend on cell surface expression of $\alpha 5\beta 1$ (Welch et al.1990).

The mechanisms by which integrin molecules transduce mechanical signals are largely unknown. One possibility is through force-dependent release of chemical second messengers, but alternatively, responses could involve changes in cytoskeletal organisation. Different cytoskeletal associations could affect positioning of the cell's metabolic machinery and/or nuclear structure.

1.8 THE ROLE OF CELL-MATRIX INTERACTIONS IN THE REGULATION OF HUMAN BONE CELL FUNCTION

Very little information is available concerning the role of the ECM and its corresponding cell surface receptors in the regulation of human bone cell function. However, over 90% of bone is composed of ECM and resident cell populations do not rely solely on cellular interactions for their phenotypic control. The potential importance of bone ECM can be illustrated *in vivo*: if devitalised and decalcified bone powder is implanted into syngeneic hosts, bone formation is induced (Reddi and Anderson, 1976). In this experimental model, bone powder is considered to promote an outgrowth of mesenchymal cells which differentiate into chondrocytes. Blood vessels subsequently invade the area, cartilage cells are replaced by osteoblasts and osteoclasts, and marrow spaces containing haemopoietic cells are observed. The newly formed osteoblasts actively synthesize bone matrix and this later becomes mineralised.

The ECM of bone is composed of at least 200 different proteins (Delmas et al.1984) many of which have yet to be fully characterised. Some of these ECM proteins e.g collagen, fibronectin and laminin are not unique to bone and have previously been shown to directly affect the actions of other cell types (reviewed by von der Mark et al.1992). Other non-collagenous ECM proteins are more bone-specific but the precise functions of these molecules are largely unknown. In addition to exerting direct effects on cell phenotype, the ECM is also concerned with the presentation of cytokines and growth factors to neighbouring cells. This increases their effective local concentration and ensures that the appropriate cytokine is available at the right time (reviewed by Gowen, 1991 and Nathan and Sporn, 1991). For example, GM-CSF, interleukin-3

and fibroblast growth factor bind heparan sulphate; TGFβ binds to biglycan and decorin; BMP3 specifically binds to collagen IV and IGF-II binds tightly to hydroxyapatite via a specific bone matrix associated binding protein.

Attachment of cells to their ECM is mediated, at least in part, through the integrins. By binding to different ligands, the cell surface expression of integrin subunits determines the microenvironment immediately surrounding the cell. Through specific cytoskeletal associations, integrin subunits potentially determine the way in which a cell responds to its external environment. In addition to this fairly passive role, the activities of many integrin molecules can be radically modulated by cells, and they, in turn, can modulate cell activities in ways that extend far beyond adhesion.

To date, bone cell research has largely concentrated on the roles of hormones, cytokines and other soluble mediators in the control of bone cell function. However, integrin-mediated interactions between bone cells and their ECM could play an important role at many different stages of bone remodelling. For example, initiation of bone resorption requires attachment to the bone matrix and formation of multinucleated osteoclasts requires fusion of mononuclear precursor cells. The process of bone resorption requires an intimate association between the sealing zone of the osteoclast and its ECM. During the reversal phase, osteoclasts must detach from the bone surface and osteoblast precursor cells must be recruited to the resorption site and activated. Matrix deposition and the subsequent formation of the osteoblast-lining cells and osteocytes could also be influenced by mechanical and chemical signals elicted by the ECM. In view of the present confusion regarding the precise functions of soluble factors, a complementary project was proposed to investigate a possible role for the ECM and the integrins in regulating human bone cell function.

CHAPTER 2.

MATERIALS AND METHODS

2.1 MATERIALS

In this section, reagents have been divided into specific categories for quick reference. All unspecified chemicals were obtained from Sigma (Poole, UK).

2.1.1 Tissue Culture Reagents

Minimal Essential Medium (MEM), penicillin and streptomycin, glutamine, sodium bicarbonate and trypsin/EDTA were obtained from GIBCO (Paisley, UK). Foetal calf serum was purchased from Imperial Laboratories (Andover, UK). High grade charcoal (Norit GSX) used for preparing charcoal stripped serum was purchased from BDH (Lutterworth, UK). MG-63 cells were obtained from the European collection of animal cultures (Salisbury, UK) and HOS cells were purchased from the American Type Culture Collection (Rockville, M.D.).

2.1.2 Biochemicals

Phosphate buffered saline (PBS) was purchased from Oxoid (Bristol, UK). Diethanolamine, chloramine T, sodium metabisulphite, potassium iodide and acetone were obtained from BDH (Lutterworth, UK). Sephadex G25 (medium grade) was purchased from Pharmacia (Milton Keynes, UK) and protein dye binding reagent was obtained from Bio-rad Laboratories (Hertfordshire, UK). ^{125}I was obtained from Amersham International (Amersham, UK).

2.1.3 Cytokines

Human recombinant IL-1β (specific activity 5×10^7 U/mg) was a gift from Glaxo Group Research (Middlesex, UK). Human TGF-β (specific activity $1\text{-}2 \times 10^7$ U/mg) was purchased from British Biotechnology (Oxford, UK) and activated by acidification according to the manufacturer's instructions.

2.1.4 Monoclonal antibodies

Monoclonal antibody (mAb) T52/7 was generously donated by Dr. Hemler (Boston, MA) and recognises the α1 (CDw49a) subunit of integrin α1β1. MAbs raised against α3 (CDw49c), α6 (CDw49f) and β1 (CD29) subunits were obtained from Bioquote Ltd (Ilkley, UK), Serotec (Oxford, UK) and Coulter Instruments (Luton, UK) respectively. MAbs directed against α2 (CDw49b), α4 (CDw49d) and α5 (CDw49e),

αL (CD11a) and β2 (CD18) subunits were purchased from Immunotech (Birmingham,UK) and a mAb directed against the αM (CD11b) subunit was obtained from Dakopatts (High Wycombe, UK). MAb 23C6 which recognises the αV (CD51) subunit in complex with β3 (Davies et al ,1989) was generously donated by Dr. Horton (London, UK) and mAb C22 which recognises the β3 subunit (CD61) was raised in our laboratory. All mAbs were obtained as ascites of hybridoma nu/nu mice or tissue culture supernatants.

2.1.5 Immunochemicals

Purified bovine osteocalcin standard and polyclonal rabbit anti-bovine osteocalcin antibody were obtained from Biogenesis (Bournemouth, UK). Normal rabbit serum was obtained from GIBCO (Paisley, UK) and goat anti-rabbit gammaglobulin was purchased from Calbiochem (Nottingham, UK). Diff-Quik reagents were obtained from Baxter Dade AG (Dudingen, Germany). Immunochemicals used for staining procedures employing alkaline phosphatase anti-alkaline phosphatase conjugates were obtained from Dakopatts (High Wycombe, UK). Human AB serum was obtained from GIBCO and FITC coated latex beads were purchased from Flow Cytometry Standards (Research Triangle Park, NC).

2.1.6 Equipment

All tissue culture plastics were obtained from Costar (Northumberland, UK) and glass slides were obtained from Henley Ltd (Loughton, UK). Colorimetric assays were quantified using a spectrophotometer (Dynatech model MR5000) and fluorimetric data were analysed using a Shimadzu spectrofluorimeter (model RF-540). Iodinated protein was monitored in LP4 tubes purchased from Luckham (Burgess Hill, UK) using an automatic gamma counter (LKB Wallac 1277 gammamaster). Cells were centrifuged onto glass slides using a Shandon cytospin, sections of osteophytic tissue were cut using a Bright's cryostat (Bright's Instruments Co.; Huntingdon, UK) and immunocytochemical studies were analysed using a Leitz microscope (Laborlux S). Cells prepared for FACS experiments were analysed on a Becton Dickenson Facstar Plus with 100 mW argon ion laser and Consort 32 computer.

2.2 TISSUE CULTURE

2.2.1 Isolation of primary human osteoblast-like cells

Trabecular bone fragments (approx. 3 mm^3) were extracted from femoral heads obtained from orthopaedic surgery (usually above knee amputation, total hip replacement or fractured neck of femur). The fragments of bone were washed extensively in PBS to remove blood and any surrounding connective tissue and then incubated overnight at 37°C in serum free medium. Bone fragments were then transferred into 9 cm diameter tissue-culture grade petri-dishes and 10 ml medium containing serum was added. Explants were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°C and old medium (10ml) was removed from the cultures and replaced once a week. Under these conditions confluency was usually achieved after 4-6 weeks. Bone cell cultures were maintained in Eagles Minimal Essential medium (MEM) containing 10% heat inactivated foetal calf serum (FCS), 100 i.u/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. FCS was heat inactivated by incubation at 56°C for 40 min; this removes complement components which could cause cell lysis.

The cells obtained from these explant cultures have been characterised previously and possess an osteoblastic phenotype. This includes the synthesis of type I collagen, alkaline phosphatase and osteocalcin (Beresford et al.1984b; Beresford et al.1986) and the production of cAMP in response to PTH (MacDonald et al.1984).

2.2.2 Culture of osteosarcoma cell lines

MG-63 and HOS TE85 cells were cultured under similar conditions to those described for bone-derived cells. However, culture medium was replaced every 3-4 days and confluency was typically achieved within a week.

2.2.3 Subculturing human osteoblast-like cells and osteosarcoma cell lines

Bone-derived cells were subcultured by replacing bone chips from trypsinised cells back into their original 9 cm diameter dishes. Fresh culture medium was added (10 ml) and explants were fed once a week as described. These cells could only be subcultured once using this method. Osteosarcoma cell lines were maintained by placing a small volume of a trypsinised cell suspension into 10 ml fresh culture medium which was

subsequently replaced every 3-4 days. The number of cell passages was noted and cell lines were subcultured up to about passage 30.

2.2.4 Passaging cells for experimental assay

MG-63, HOS TE85 and human osteoblast-like cells were passaged into 9 cm diameter tissue culture grade petri-dishes, multi-well plates or onto glass slides by trypsinisation. At confluency, the medium was removed, cell layers were washed with 10 ml sterile PBS to remove traces of serum and 2.5 ml/plate trypsin/EDTA solution (0.25% w/v trypsin, 0.2% w/v EDTA) was added. After 5 min at 37°C for osteosarcoma cell lines and 10-15 min at 37°C for bone cell cultures, cells were detached and further trypsinisation was prevented by the addition of 7.5 ml fresh medium containing serum. Cells were pelleted by centrifugation (1000 rpm/5 min), resuspended in fresh culture medium and counted using a haemocytometer. Cells were then seeded into assay plates at the required density and used for experimental purposes.

2.2.5 Detaching cells from assay plates

In order to detach cells from their ECM, medium was removed and cell layers were washed with PBS (1 ml/well for 24-well plates and 5 ml/plate for 9 cm dishes). Trypsin/EDTA solution was then added to cells grown on plastic, fibronectin, collagen I film and laminin (250 µl/well for 24 well plates and 2.5 ml/plate for 9 cm dishes) and collagenase I (0.3% w/v in serum free MEM) was added to cells grown on collagen I gels (the volume of collagenase added was equivalent to the volume of the gel). After 5 min in trypsin/EDTA and 30 min in collagenase, trypsin was neutralised by the addition of MEM+10% FCS (750 µl/well for 24 well plates and 7.5 ml/plate for 9 cm dishes) and cells were collected by centrifugation (6 500 rpm/5 min in a microfuge or 1000 rpm/5 min in a bench top centrifuge).

2.3 ROUTINE PREPARATIONS

2.3.1 Preparation of charcoal stripped serum (CS-FCS)

100 ml heat inactivated serum was stirred with 4 g high grade charcoal (Norit GSX) on ice for 1 hr. Charcoal was separated from serum by centifugation (3000 rpm/15 min) and filtration through 0.4 µm pre-filters. Serum was then re-sterilized using 0.2 µm filters and stored in aliquots at -20°C. The main function of this procedure is to remove steroids present in serum.

2.3.2 Preparation of rat tail collagen (Type I)

Type I collagen was prepared from rat tails using a method based on a report by Strom and Michalopoulos (1982). 12 rat tails were sterilised by repeatedly dipping them into industrial methylated spirits. Tendons were then dissected from the tails using forceps, transferred into 0.1% acetic acid (500-1000 ml) and stirred at 4°C for 48 hr. The resulting solution was filtered through absorbant gauze and separated by centrifugation (10 000 rpm/30 min/4°C). 1 M NaOH was then added to the supernatant in a ratio of 1: 6 to precipitate the collagen. This collagen precipitate was collected by centrifugation (2500 rpm/10 min), resuspended in a minimal volume of 0.1% acetic acid and the concentration of collagen was determined by dry weight. Stock solutions (1 mg/ml) were then prepared in 0.1% acetic acid and stored at 4°C until required.

2.3.3 Preparation of collagen I gels

Collagen I gels were prepared by a method used at Glaxo Group Research. A stock solution of concentrated medium (see below) was added to wells containing collagen type I in a ratio of 1.33:1. The contents were mixed well and the plates were incubated at 37°C for 30 min. During this time the increased pH caused the collagen to form a gel which stabilised after a few hours. Gels were stored overnight at 4°C and then used for experimental purposes.

Preparation of concentrated medium:

10 X MEM	6 ml
Penicillin (10 000 i.u/ml)/Streptomycin (10 000 µg/ml) solution	0.25 ml
Glutamine (200 mM)	0.25 ml
CS-FCS	12 ml
0.1 M sterile NaOH	7 ml

2.4 BIOCHEMICAL AND CELLULAR ASSAYS

2.4.1 Measurement of cell proliferation

MG-63, HOS TE85 and human osteoblast-like cells were cultured under test conditions for the required number of days. At the end of the assay period, cells were detached by trypsinisation or treatment with collagenase (Method 2.2.5) and collected by

centrifugation. Cell pellets were then resuspended in MEM+10% FCS, stored on ice to minimise cell clumping and 2 x 10 µl aliquots were counted in a haemocytometer.

2.4.2 Alkaline phosphatase assay

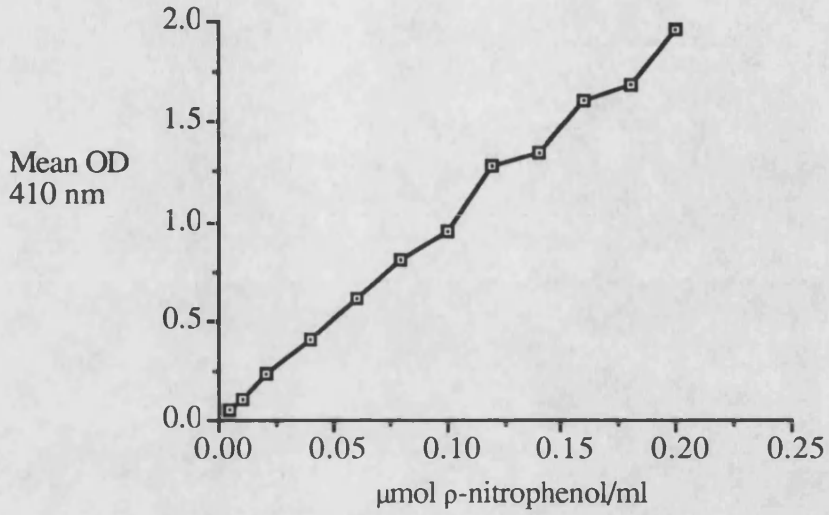
Cellular alkaline phosphatase acivity was measured colorimetrically in detached cells by monitoring the release of para-nitrophenol (ρ-nitrophenol) from disodium ρ-nitrophenyl phosphate at 37°C and pH 10.5. The reaction produces a coloured product which can be measured spectrophotometrically at 410 nm.

MG-63, HOS TE85 and human osteoblast-like cells were cultured under test conditions for up to 10 days. At the end of the treatment period, the medium was removed (and retained for osteocalcin assay if required) and cells were detached by trypsinisation or treatment with collagenase (Method 2.2.5): neither of these enzymes interfered with alkaline phosphatase activity (see Chapter 3). When cells were to be simultaneously assayed for cell number or DNA, cell suspensions were divided in a ratio of 1:2 and the larger aliquot was assayed for alkaline phosphatase. Cell pellets were resuspended in 100 µl Tween 20 (0.1% in de-ionised H_20) and 3 x 25 µl aliquots were transferred into wells of a 96-well plate. The assay plate also contained blanks (25 µl/well 0.1% Tween 20) and known concentrations of ρ-nitrophenol standard (25 µl/well). Standards were diluted in 0.1% Tween 20 from a 10 mM stock to give concentrations of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 µmol/ml. 225 µl aliquots of substrate buffer was added to all wells (0.1 M diethanolamine, 0.5 M $MgCl_2$, pH 10.5 and 46 mg/100ml 4-disodium-ρ-nitrophenyl phosphate) and the assay plate was incubated at 37°C. After 30-40 min, or when colour intensities from samples were similar to those of the lower standards, the absorbance was recorded at 410 nm. Enzyme activity, expressed as µmol/ml ρ-nitrophenol/hr was then determined from a standard curve (Fig. 2.1).

2.4.3 DNA assay

A DNA assay was developed from a method used by Mates et al.(1986) in order to obtain an accurate measurement of cell number. In this assay, cellular DNA was quantified using the Hoechst stain 33258. This compound specifically intercalates with DNA and produces a linear increase in fluorescence with cell number. This method was mainly used in conjunction with the alkaline phosphatase assay so that enzyme activity could be related to cell number. All samples were assayed immediately as storage at 4°C or -20°C interferes with fluorescence values.

Figure 2.1 A typical standard curve obtained from an alkaline phosphatase assay



2.4.3.1 Optimisation of reagent concentrations

Initially, a series of standard DNA solutions containing 0.00-1.0 µg DNA were prepared in TRIS buffer (10 mM TRIS, 10 mM EDTA, 10 mM NaCl, pH 7.0). 1.0 ml EDTA (10 mM, pH 12.3) was added to each standard followed by 100 µl KH_2PO_4 (1 M): the pH of this reagent was adjusted so that when 10 µl was added to 100 µl EDTA, the pH was 7.0. 1 ml Hoechst 33258 (80 µg/ml in TRIS buffer) was then added to all standards and the fluorescence of each DNA standard was recorded over an excitation range of 250-500 nm. Fluorescence readings obtained from the DNA standards were almost identical (data not shown) implying interference from one or more reagents.

As a result of this finding, the emission profile of each reagent was assessed and the volume producing least interference was determined.

Optimised reagent volumes are shown below:

Calf thymus DNA (single stranded)	0.05-1 µg in 200 µl TRIS buffer
EDTA	100 µl
KH_2PO_4	10 µl
Hoescht solution (80 µg/ml)	500 µl
TRIS buffer	1400 µl

Excitation and emission profiles from this solution showed optimum excitation and emission wavelengths of 354 nm and 473 nm respectively.

A typical standard curve for DNA is shown in Figure 2.2A. The assay was linear over a range of 0.025-1 μg DNA which was appropriate for assessing DNA content of osteoblast-like cells seeded into 24-well plates.

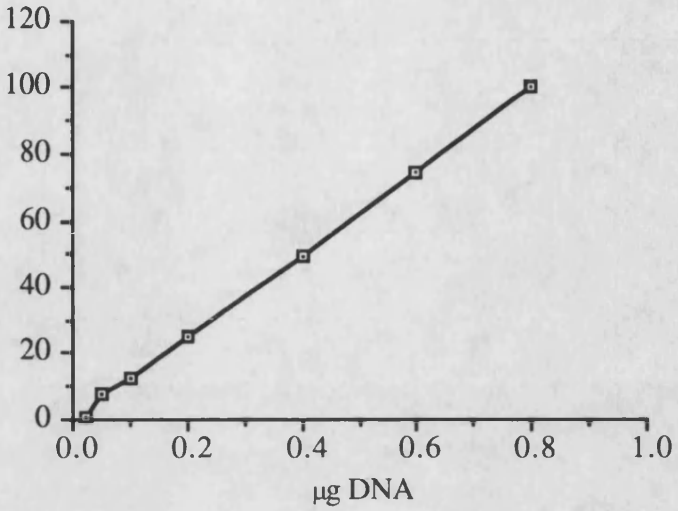
2.4.3.2 Linearity of DNA assay with cell number

Before this procedure was used as an assessment of cell density, the linearity with cell number was determined using a range of concentrations of WEHI cells. This mouse leukaemic cell line was used in preference to human osteoblast-like cells due to their relative abundance. Figure 2.2B illustrates that the assay was linear over a range of 1 250-20 000 cells. These results produced a mean value of 29 pg DNA/cell which agrees with data obtained previously (Mates et al.1986).

2.4.3.3 Measurement of cellular DNA

MG-63, HOS TE85 and human osteoblast-like cells were cultured in 24-well plates for up to 10 days. At the end of the treatment period, the medium was removed (and retained for osteocalcin assay if required) and cells were detached (Method 2.2.5) and divided in a ratio of 1:2. Cells from the smaller aliquot were then washed in PBS (1 x 1 ml) to remove any residual medium (phenol red interferes with the DNA assay) and lysed by the addition of 100 μl EDTA. Blanks (200 μl TRIS buffer) and standards (200 μl in TRIS buffer) were also placed under these conditions. Standards were prepared from calf thymus DNA (Type 1; stored at 4°C) to give 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0. 1.2, 1.4 and 1.6 μg DNA/tube. After 20 min at 37°C, 10 μl KH_2P0_4 was added, followed by TRIS buffer; 1600 μl to samples and 1400 μl to standards and blanks. Finally, 500 μl Hoechst 33258 solution (80 μg/ml in TRIS buffer) was added to all tubes and the fluorescence was measured in a quartz cuvette using a fluorimeter. The excitation and emission wavelengths were set at 354 nm and 473 nm respectively. Fluorescence of all samples was recorded in triplicate and cellular DNA was then determined from mean values using the standard curve.

Figure 2.2A



Fluorescence units

Figure 2.2B

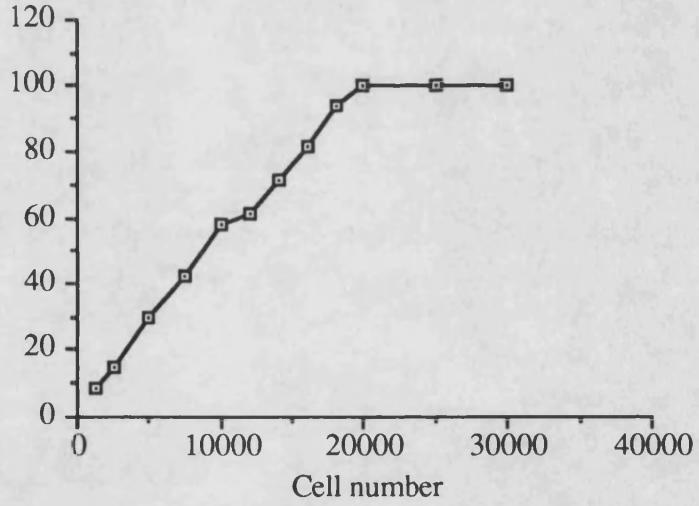


Figure 2.2. Evaluation of a DNA assay for assessment of cell number.

A typical standard curve obtained from the DNA assay (A). Linearity of DNA assay with respect to cell number (B). A series of DNA standards prepared in 200 µl Tris buffer and dilutions of WEHI cells were incubated with EDTA (20 mins/37°C). 10 µl KH_2PO_4 was then added followed by Tris buffer (1 400 µl to standards; 1 600 µl to cells). Finally, 500 µl Hoechst 33258 was added to all samples and the fluorescence was recorded (excitation = 354 nm; emission = 473 nm)

2.4.4 Osteocalcin assay

Iodination of osteocalcin

Osteocalcin standard was resuspended in 40 µl PBS and transferred to a reaction vessel containing a magnetic flea. 10 µl of ^{125}I (1 mCi) was then added followed by 20 µl chloramine T (0.5% w/v) which started the reaction. After exactly 1 minute, the iodination was terminated by the addition of 200 µl sodium metabisulphite (0.016% w/v) followed by 400 µl KI (1% w/v). The contents of the reaction vessel were transferred to the top of a Sephadex G25 column (prepared by incubating 5 g Sephadex medium grade G25 with 100 ml PBS+1% BSA overnight at 10°C) and ^{125}I-osteocalcin was eluted with PBS+1% BSA. 40 x 1 ml fractions were collected and 5 µl aliquots were counted in a gamma counter. The hottest fractions containing iodinated protein were pooled, diluted in Bent T (0.01 M TRIS pH 7.4, 0.14 M NaCl, 0.025 M EDTA, 0.1% Tween 20, 0.25% BSA) to 10 000 cpm/50 µl, and stored at -20°C until required.

Osteocalcin assay

Osteocalcin released into supernatants was measured by specific competitive radioimmunoassay (RIA). In this assay, radiolabelled osteocalcin (^{125}I-osteocalcin) and cold osteocalcin in standards or samples compete for binding sites on a primary antibody (rabbit anti-bovine osteocalcin). A second antibody (goat anti-rabbit gammaglobulin) is then added to precipitate the osteocalcin-primary antibody complex and this is collected by centrifugation and counted at the end of the assay.

Supernatants were collected from human bone-derived cells or osteosarcoma cell lines and 3 x 50 µl aliquots were transferred into LP4 tubes. Reference tubes detecting total counts, non specific binding and maximum binding were then prepared in triplicate. Total counts (TC) were determined by the addition of 3 x 50 µl aliquots of ^{125}I-osteocalcin. Non-specific binding (NSB) was detected using 3 x 50 µl aliquots of 2 % normal rabbit serum (NRS) diluted in Bent T. Maximum binding (MAX) was assessed by recording counts obtained in the absence of cold osteocalcin. A standard curve of purified bovine osteocalcin was then prepared in triplicate by serial dilution to give standards of 100, 50, 33, 20, 10, 5, 3.3, 2, and 1ng/ml. 50 µl primary antibody (diluted 1/10 in Bent T) was then added to all tubes except TC and NSB. Next, 50 µl 2% NRS was added to all tubes except TC, to produce a larger more visible pellet, followed by 50 µl ^{125}I-osteocalcin. The total volume was made up to

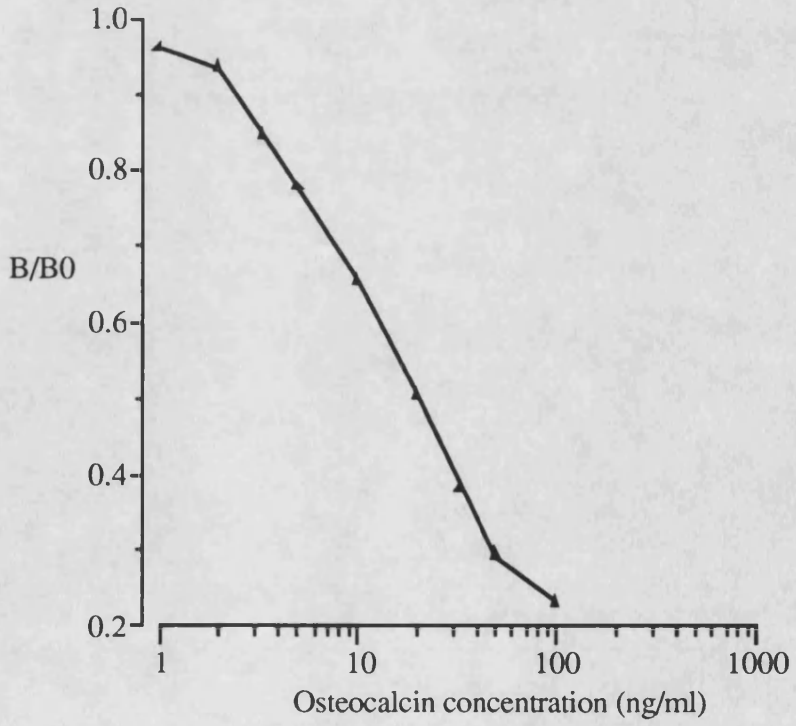
300 µl using Bent T (except TC), the contents were mixed well and all tubes were incubated overnight at 4°C. After this time, 50 µl secondary antibody (diluted to 20 ml with Bent T), 50 µl polyethylene glycol (5% in H_20; mw 8000) and 500 µl Bent T was added to all tubes except TC. Tubes were mixed again and after a further overnight incubation at 4°C, precipitates were collected by centrifugation at 3000 rpm/ 25 min/ 4°C. The supernatants were removed by aspiration (not TC) and the amount of radioactivity in all tubes was monitored using a gamma counter.

A graph of $\log_{10}$ concentration (abscissa) vs B/B_0 (ordinate) was then plotted where B/B_0 is calculated from the following equation:

$$B/B_0 = \frac{\text{Counts per min. (standard/sample)-NSB}}{\text{MAX-NSB}}$$

A typical standard curve is shown in Figure 2.3. Osteocalcin released into supernatants by bone cell cultures and osteosarcoma cell lines was determined using this curve and values were expressed as ng/well of conditioned medium.

Figure 2.3 A typical standard curve for the osteocalcin assay



2.4.5 Growth of human osteoblast-like cells on different matrix components

24-well plates or 9 cm diameter tissue culture grade petri-dishes were coated with different concentrations of purified matrix components. Solutions of fibronectin (0.1-50 $\mu g/cm^2$), collagen I (0.1-50 $\mu g/cm^2$) and laminin (0.1-100 $\mu g/cm^2$) were prepared by diluting stock solutions (1 mg/ml) in sterile PBS; 1 ml was added to wells of 24-well plates and 5 ml was added to 9 cm diameter dishes. Collagen I gels were prepared was described above (Method 2.3.3); 0.25-1 ml volumes were added to wells of 24 well plates and 10 ml volumes were added to 9 cm dishes. All experiments were performed in the presence of a control where wells were coated with sterile PBS; 1 ml was added to wells of 24-well plates and 5 ml was added to 9 cm diameter dishes. All coated plates and dishes were incubated overnight at 4°C to allow maximum attachment of fibronectin, collagen I film and laminin, and to ensure optimal equilibration of collagen I gels.

Following an overnight incubation, excess coating was removed and all wells/dishes were washed three times with PBS (1 ml/well for 24-well plates and 5 ml/plate for 9 cm dishes). Confluent cultures of human osteoblast-like cells were then trypsinised (Method 2.2.4) and plated onto purified matrix components in MEM+10% CS-FCS. 24-well plates were seeded at 40 000 cell/well (1 ml/well) and 9 cm dishes were seeded at 600 000 cells/plate (10 ml/plate). 50 % of the medium was replaced every 2 days for the next 10 days to ensure a good supply of nutrients. Cells cultured in 24 well plates were assayed for changes in differentiated osteoblast phentoype. Therefore medium was removed 72 hr prior to the end of the treatment period, cells were washed with serum free medium (1 ml/well) and MEM+5% CS-FCS containing vitamin K (10^{-8}M) and vitamin C (50 $\mu g/ml$) was replaced (500 μl/well). Half the cells also received 1,25D (10^{-8}M), a standard differentiating agent used to promote alkaline phosphatase activity and osteocalcin production, and to decrease proliferation (Skjodt et al.1985; Beresford et al.1984b; Beresford et al.1986); the remainder were treated with vehicle alone. Cells to be assayed for changes in integrin subunit expression by FACS analysis were not stimulated with 1,25D but maintained in MEM+10% CS-FCS throughout the treatment period.

After a total of 10 days on different matrix components, cells in 24-well plates and 9 cm diameter dishes were assayed for changes in osteoblast phenotype.

2.4.6 Treatment of human osteoblast-like cells and osteosarcoma cell lines with 1,25D, IL-1β and TGFβ

Confluent cultures of MG-63, HOS TE85 and human osteoblast-like cells were seeded into 9 cm diameter petri-dishes or 24 well plates in MEM+10 % CS-FCS (Method 2.2.4). 9 cm dishes were seeded at 600 000 cells/plate (10 ml/plate) and 24-well plates were seeded at 40 000 cells/well (1 ml/well). After an overnight incubation, the medium was removed, cells were washed in serum free medium and treatments were applied (5 ml/9 cm dish; 500 µl/24-well plate). In order to study the effects of 1,25D, experiments were performed in MEM+5% CS-FCS supplemented with vitamin K (10^{-8}M) and vitamin C (50 µg/ml): 10^{-8}M 1,25D was added to half of the cells and the remainder received vehicle alone. Experiments investigating the effects of IL-1β and TGF-β were undertaken in MEM+3% CS-FCS. IL-1β was used at a concentration of 10 U/ml and TGF-β was used at 25 ng/ml; test agents were added to half the wells and the remainder received vehicle alone. All cells were incubated under test conditions for up to 72 hr and at the end ot the treatment period, control and treated cells were assayed for modulation of osteoblast phenotype.

2.5 IMMUNOCYTOCHEMISTRY

2.5.1 Preparation of cryostat sections and cultured cells

Cryostat sections were cut from osteophytic bone isolated from femoral heads following orthopaedic surgery or from osteoclastoma tissue. Both tissues were dipped briefly into polyvinyl alcohol and chilled at -70°C by precipitate immersion in n-hexane (Chayen et al.1973). 8 µm sections of the tissues were then cut on a Bright's cryostat, fixed with acetone (10 min) and stored at -70°C until required.

Cells used for immunocytochemical studies were detached from confluent plates by trypsinisation and seeded onto 10 well multi-spot slides at 2000 cells/well in growth medium (50 µl/well) (Method 2.2.4) . Cells were cultured for 72 hr in a humidified box to prevent evaporation. At the end of the incubation period, the medium was removed, slides were washed in PBS and cells were fixed in acetone (10 min). Slides were air dried, wrapped in foil and stored at -70°C for up to 3 months.

2.5.2 Histology

8 μm cryostat sections of acetone fixed undecalcified human osteophyte were stained using Diff-Quik. This reagent consists of two solutions, Eosin G (solution 1) and Thiazine (solution 2), which when added consecutively, stain newly formed non-mineralised osteoid pink and cells blue; mineralised bone remains unchanged.

2.5.3 Immunolocalisation procedures

All steps were performed at room temperature in a moist chamber to minimize evaporation. At each stage, the area around the edge of the well was carefully dried with a tissue to prevent inter-well carry over: 25 μl of reagent was added to wells of 10 well multi-spot slides and 80 μl was added to wells of 4 well multi-spot slides. All studies were performed in the presence of an appropriately diluted negative control and if no staining was detected, the functionality of the mAb was assessed using a cell type known to express the subunit in question.

2.5.4 Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique

This procedure was essentially performed according to the manufacturer's protocol (Dakopatts). Acetone fixed cells and sections were overlayed with mouse mAb or mouse IgG (negative control) diluted appropriately in TRIS-buffered saline (TBS; 0.05 M TRIS-HCl pH 7.6, diluted 1/10 in 0.15 M isotonic saline). After 30 min, slides were washed for 1-2 min in TBS and anti mouse IgG (Dakopatts code no. Z 259 diluted 1/25 in TBS) was added. After a further 30 min, slides were washed again and APAAP complex (Dakopatts code no. D 651 diluted 1/50 in TBS) was replaced. After a further 30 min, slides were washed for a third time and flooded with alkaline phosphatase substrate. Different substrates were used for sections and cells (see below) but in both cases 4 mM levamisole was added to block endogenous alkaline phosphatase activity. This is four times higher than that recommended in the manufacturer's protocol but was found to be the optimum concentration by previous titration. After 15-20 min the reaction was terminated by immersing slides in TBS and then in tap water. Cells and sections were then lightly counterstained with Mayer's Haematoxylin for 30 sec. Excess stain was removed with tap water and the slides were mounted in 90% glycerol in PBS. After sealing slides with nail varnish, specimens were examined microscopically.

Alkaline phosphatase substrate 1 (for acetone fixed cells)

Naphthol AS-MX phosphate	2 mg
Dimethylformamide	0.2 ml
0.1 M TRIS buffer pH 8.2	9.8 ml
1M levamisole	40 µl
Fast-Red TR salt	10 mg

Naphthol AS-MX phosphate was dissolved in dimethylformamide, diluted to 10 ml with TRIS buffer (pH 8.2) and levamisole was added. Immediately before staining, the Fast-Red salt was added to the substrate solution and filtered directly onto glass slides.

Alkaline phosphatase substrate 2 (for acetone fixed sections)

Naphthol AS-BI phosphate	5 mg
Dimethylformamide	60 µl
0.05 M TRIS buffer pH 8.7	10 ml
1M levamisole	40 µl
4% sodium nitrite (freshly prepared)	50 µl
5% New Fuchsin in 2 M HCl	20 µl

5% New Fuschin solution was mixed with 4% sodium nitrite in a fume hood for 30-60 sec and TRIS buffer and levamisole were then added. Naphthol AS-BI substrate was dissolved in dimethylformamide and added to the above solution. The reagents were mixed well and then filtered directly onto glass slides.

2.5.5 Immunofluorescence (IF)

A standard indirect immunofluorescence procedure (Johnson, 1981) was employed for immunolocalisation of α6 because mAb GoH3 is raised in rats. Slides were washed for 30 min in PBS / 2% FCS (washing buffer) and overlayed with mAb or rat IgG (negative control) diluted appropriately in washing buffer. After 30 min, slides were washed again and FITC-conjugated rabbit anti-rat immunoglobulin (diluted 1/100 in washing buffer) was applied. The secondary antibody was preabsorbed with 1% human AB serum (diluted in washing buffer) for 2 hr and immunoprecipitate was collected by centrifugation (10 000 rpm/5 min in a microfuge); this prevents non specific binding to human proteins. After a further 30 min, slides were washed in PBS

alone and cells and sections were counterstained for 30 sec by immersion in 0.002% ethidium bromide in PBS. Residual dye was removed by rapidly rinsing the slides twice in PBS and then washing them for a further 30 min. Slides were mounted using 90% glycerol in PBS containing 2.5% DABCO (diaza-bi-cyclo-octane); this prevents quenching of fluorescence. After sealing with nail varnish, slides were examined microscopically.

On some occasions, a high background fluorescence was observed which resulted from non specific binding. This could be reduced by incubating cells and sections with rabbit serum (diluted 1/5 in washing buffer) for 20 min at the beginning of the procedure.

2.6 PREPARATION OF CELLS FOR FLOW CYTOMETRY (FACS ANALYSIS)

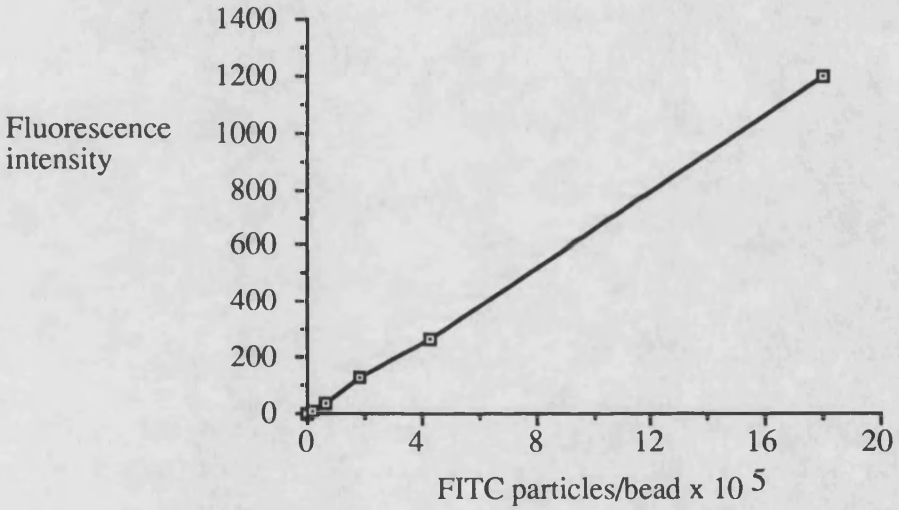
MG-63, HOS and human osteoblast-like cells were seeded into 9 cm diameter petri-dishes in MEM+10 % FCS (Method 2.2.4) and incubated under test conditions for up to 10 days. At the end of the treatment period, the medium was removed (and retained for osteocalcin assay if required) and cells were detached by trypsinisation or treatment with collagenase (Method 2.2.5): neither of these enzymes interfered with integrin subunit expression (see Chapter 5). Cell pellets were then resuspended in 5 ml growth medium and 2 x 10 µl aliquots were counted in a haemocytometer. 1 x 10^5 cells were transferred into FACS tubes and resuspended in 60 µl of mAb or IgG (negative control), appropriately diluted in PBS containing FCS (1%) and human AB serum (1:5 dilution). After 90 min on ice with occasional agitation, cells were washed in 4 ml dilution buffer (PBS+1% FCS) and resuspended in 50 µl of the appropriate FITC-conjugated secondary antibody (diluted 1/20 in dilution buffer). After a further 30 min on ice, cells were washed again, fixed in 500 µl paraformaldehyde (1% in PBS) and stored at 4°C until required. Integrin subunit expression was assessed by passing samples through a Becton Dickinson Facstar plus and collecting and analysing 5000 events using the program LYSIS II.

2.6.1 Analysis of FACS data

The program LYSIS II gave measurements of side scatter (SSC), forward scatter (FSC) and mean fluorescence intensity (MFI). When analysing FACS data, values obtained for the negative control (i.e cells incubated in the presence of IgG) were used to produce a dot plot of SSC against FSC. These parameters gave an assessment of

granularity and size, and were used to determine the homogeneity of the sample population. Cell preparations were typically slightly heterogeneous and so a specific region enclosing the majority of the population was selected and used for subsequent analysis. For detection of integrin subunit expression, MFI values obtained from all samples (ordinate) were plotted against cell number (abscissa) and data from negative controls were overlayed with data obtained using anti-integrin mAbs. When quantifying receptor sites/cell, MFI values obtained from preparations of 6 standardised FITC-coated fluorescent beads were used to plot a standard curve of fluorescence against number FITC particles/bead (Fig. 2.4): values for sites/cell were then determined by regression.

Figure 2.4 A typical standard curve of FITC particles/bead against fluorescence for quantifying receptor sites/cell



2.7 ATTACHMENT ASSAYS

96-well flat bottomed plates were coated with PBS (control), fibronectin (0.001-5 μg/cm^2), collagen I (0.001-5 μg/cm^2) and laminin (0.01-20 μg/cm^2). Different concentrations of ECM proteins were prepared by diluting stock solutions (1 mg/ml) in PBS and100 μl was added to each well. After an overnight incubation at 4°C to allow optimal attachment of matrix proteins, excess coating was removed and wells were washed 3 x 100 μl/well PBS. Non specific binding was then blocked by the addition of 100 μl/well BSA (2 mg/ml in PBS). After 1 hr at room temperature, wells were washed again (3 x 100 μl/well PBS) and cells were seeded into wells at 15 000 cells/well in serum free MEM (100 μl/well). Therefore, confluent cell layers were detached by trypsin (Method 2.2.4), washed in serum free medium (2 x 10 ml) to

remove any traces of serum, counted and seeded into 96 well plates coated with ECM proteins (or PBS). Plates were incubated at 37°C for 2 hr and % adhesion was assessed using a Bradford protien assay (Bradford, 1976). This colourimetric assay was performed using a kit from Biorad Laboratories and is based on a differential colour change in response to varying concentrations of protein. More specifically, the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm as binding to protein occurs.

In order to measure attached cell protein, the medium was carefully removed, cells were washed with 1 x 100 µl/well PBS and then lysed by the addition of 200 µl/well de-ionised H_20. 50 µl/well protein dye reagent was then added, wells were mixed and after 5 min the absorbance was recorded at 630 nm. All samples were assayed at least in triplicate and OD readings were subtracted from values obtained from blank wells containing ECM protein alone. In order to assess non attached cell protein, medium from supernatants and PBS from cell washes were pooled and retained. Cells within these fractions were collected by centrifugation in a microfuge (6 500 rpm/5 min) and resuspended in de-ionised H_20 (Typically the contents from three wells were pooled and resuspended in 600 µl H_20). 200 µl aliquots were dispensed into wells of a 96-well plate and 50 µl/well Biorad dye reagent was added. After mixing well, cell lysates were left to stand for 5 min and the absorbance was recorded at 630 nm. % adhesion was determined using the following equation:

$$\% \text{ adhesion} = \frac{\text{OD from attached cells}}{\text{OD from attached + non attached cells}} \times 100$$

CHAPTER 3.

MODULATION OF OSTEOBLAST PHENOTYPE BY EXTRACELLULAR MATRIX COMPONENTS

3.1 Abstract

The aim of this study was to investigate the effects of three different ECM proteins on the phenotypic characteristics of human osteoblast-like cells over a period of 10 days. Four of the main parameters commonly used to assess osteoblast function were examined, namely morphology, proliferation, alkaline phosphatase activity and osteocalcin production. All studies were undertaken in the presence and absence of 1,25D, a bone-derived cell differentiating agent, and results obtained were compared to those observed on tissue culture plastic.

Culture on different concentrations of fibronectin (0.1-50 $\mu g/cm^2$) for 10 days did not significantly affect cell morphology or alkaline phosphatase activity, but it slightly stimulated proliferation and decreased 1,25D induced osteocalcin release. Growth on different concentrations of laminin (1-100 $\mu g/cm^2$) or type I collagen (0.1-50 $\mu g/cm^2$) for the same time period did not affect morphology. However, both of these ECM components decreased cellular alkaline phosphatase activity and 1,25D induced osteocalcin production. The effects of collagen I film were generally more pronounced than those of laminin and in addition, this matrix caused a reduction in proliferation which was not apparent following growth on laminin. Culture of human osteoblast-like cells on collagen gels (1.25-5 mm depth) exerted the most dramatic effects. This matrix induced a change in cell morphology and decreased cell proliferation, alkaline phosphatase activity and 1,25D induced osteocalcin release.

The effects of culturing human osteoblast-like cells on different ECM components are compared with results obtained using other osteoblast models and non-bone cell types. In addition, the relevance and possible implications of these findings *in vivo* are discussed.

3.2 Introduction

The ECM may be considered as an insoluble local mediator which plays an important role in regulating many different aspects of cell function. The ECM of bone is composed of at least 200 different proteins (Delmas et al.1984) many of which have yet to be fully characterised. Some of these proteins are the secretory products of osteoblasts and others are derived from the vascular basement membrane. The aim of these studies was to determine whether three of these ECM components, namely collagen I, fibronectin and laminin, affect the phenotype of cultured human osteoblast-like cells. Collagen I, the most abundant protein in the bone matrix, can influence cell polarity, morphological organisation and gene expression of mouse mammary epithelial cells. In addition, it can influence the transformation of mesenchymal lens epithelium and promote capillary duct formation of endothelial cells (reviewed by von der Mark et al.1992). Fibronectin is one of the most prevalent and versatile of the non collagenous bone matrix proteins and can promote attachment and spreading of mesenchymal and epithelial cells. It can also influence the proliferation and migration of embryonic tumour cells and can control cell differentiation, cell shape and cytoskeletal organisation (reviewed by von der Mark et al.1992). Laminin is a major adhesive glycoprotein of cell basement membranes and can control a number of biological activities. For example, it induces polarisation and differentiation of melanoma cells, endothelial cells and mouse mammary epithelial cells. It also stimulates neurite growth and enhances proliferation and motility of tumour cells and skeletal myoblasts. Angiogenesis is an essential prerequisite for bone formation (Trueta, 1963; Froidart and Reddi, 1980). Since osteoblasts and osteoprogenitor cells are in contact with the basement membrane of the invading vascular system, it has been suggested that the endothelial cell matrix, which is rich in laminin, may play a role in regulating bone cell function (Vukicevic et al.1990).

Methods for investigating the long term effects of ECM components on human osteoblast phenotype were developed from a number of different reports. Fibronectin and collagen I film were applied over a concentration range of 0.1-50 $\mu g/cm^2$ and laminin was used over a range of 1-100 $\mu g/cm^2$. This range encompasses those concentrations used by other workers studying the effects of ECM proteins on non bone cell types (Kleinman et al.1981; Ruoslahti et al.1982; Aggeler et al.1988; Adams and Watt, 1989; Fernandez and Ben-Ze'ev, 1989; Vukicevic et al.1990). In light of the results obtained using primary cultures of mammary epithelial cells (Emerman and Pitelka, 1977; Lee et al.1984; 1985; 1987), hydrated collagen I gels were also prepared

to a depth of approximately 1.25-5 mm (0.25-1.0 ml volume in 24-well plates). Previous studies investigating the differentiating effects of ECM components were performed over a period of 6-8 days (Emerman and Pitelka, 1977; Lee et al.1984; 1985; 1987). Therefore human osteoblast-like cells were cultured on purified matrix components for 7 days and then half the cells were treated with 1,25D to induce the expression of the differentiated osteoblast phenotype. After a total of 10 days in culture, four different parameters of osteoblast phenotype were assessed, namely cell morphology, proliferation, alkaline phosphatase activity and osteocalcin production. The second two markers are indicative of a more differentiated osteoblast phenotype and when cells are grown on tissue culture plastic, levels are typically increased by 1,25D (Beresford et al.1984b; Skjodt et al.1985; Beresford et al.1986). Any modulation of osteoblast phenotype following growth on different ECM proteins was assessed in the presence and absence of 1,25D by comparing levels obtained with those observed on medical grade polystyrene tissue culture plastic.

3.3 Methods

3.3.1 Effect of trypsin on cellular alkaline phopshatase activity

Human osteoblast-like cells were cultured in two 24-well plates in the presence and absence of 1,25D (Method 2.4.6). After 72 hr, cells from one plate were assayed for alkaline phosphatase directly, and cells from the other plate were detached by trypsinisation and then alkaline phosphatase activity was assessed. Cell layers to be assayed directly were washed with PBS (1 x 1 ml) and lysed with 0.1% Tween 20 (200 µl/well). Cells from the other plate were detached by trypsinisation (Method 2.2.5), resuspended in 0.1% Tween 20 (200 µl/well) and transferred to 24-well plates. 1.80 ml substrate buffer (prepared as described in Method 2.4.2) was then added to all wells and both plates were incubated at 37°C for 30-40 min. 250 µl aliquots were transferred to wells of a flat bottomed 96-well plate and the absorbance was recorded at 410 nm. Enzyme activity was then determined from a standard curve (prepared as described in Method 2.4.2) and results were expressed as µmol ρ-nitrophenol/ml/hour (Table 3.1A).

3.3.2 Effect of collagenase on cellular alkaline phosphatase activity

Human osteoblast-like cells were cultured in two 24-well plates in the presence and absence of 1,25D (Method 2.4.6). After 72 hr, cells from one plate were trypsinised and assayed for alkaline phosphatase; cells from the other plate were incubated with

collagenase I (0.3% in serum free medium) for 30 min and 60 min, then trypsinised and alkaline phosphatase activity was assessed (Method 2.4.2). Enzyme activities were determined from a standard curve and results were expressed as μmol ρ-nitrophenol/ml/hour (Table 3.1B).

3.3.3 Effect of trypsin and collagenase on the osteocalcin assay

In order to assess the effects of trypsin, a standard curve was constructed in PBS using purified bovine osteocalcin (1-100 ng/ml; 250 μl/well). 125 μl/well trypsin/EDTA solution was then added and after 5 min at 37°C, enzyme activity was neutralised by the addition of 125 μl MEM+20% FCS. 3 x 50 μl aliquots of this solution were assayed for osteocalcin (Method 2.4.4). In order to assess the effects of collagenase in this assay, a series of osteocalcin standards were prepared (10-1000 ng/ml). 25 μl of each standard was then dispensed into wells of a 24-well plate and a collagen I gel (225 ul) was prepared on top. Collagenase I was added (0.3% in serum free medium; 250 μl/well) and after 30 min at 37°C, 3 x 50 μl aliquots were assayed for osteocalcin. These assays were performed in the presence of two other standard curves: one of these was prepared as described above and the other was prepared in Bent T as described in Method 2.4.4.

3.3.4 Effect of collagen fibres on the osteocalcin assay

An osteocalcin standard curve was constructed in PBS using purified osteocalcin standard (10-1000 ng/ml). 25 μl aliquots were then overlayed with 225 μl collagen I gels as described above. After the gels had formed, they were dispersed by drawing the contents through a syringe, and then 3 x 50 μl aliquots were assayed for osteocalcin (Method 2.4.4). A similar standard curve was prepared simultaneously in serum free medium (10-1000 ng/ml; 250 μl/well) and 3 x 50 μl were assayed for osteocalcin.

3.3.5 Effect of collagen I gels on total osteocalcin production

Human osteoblast-like cells were cultured on tissue culture plastic and collagen I gels for 10 days (Method 2.4.5). At the end of the treatment period, supernatants were removed and retained for osteocalcin assay. Collagen I gels were then overlayed with an equal volume of serum free medium and mechanically dispersed using a syringe. These samples still contained attached cells and therefore cells seeded onto plastic were scraped and resuspended in appropriate volumes of serum free medium. Another control containing dispersed collagen I gels in the absence of cells was incorporated

into the assay and 3 x 50 µl aliquots of all samples were assessed for osteocalcin (Method 2.4.4).

3.4 Results

3.4.1 Effect of matrix components on cell morphology

Cell morphology following growth on fibronectin, collagen I film and laminin was not significantly different from that observed on tissue culture plastic (Fig. 3.1A). All cells exhibited a typical flattened, fibroblastic morphology which was not affected by 1,25D. In contrast, cells cultured on collagen I gels displayed long cellular processes which penetrated the gel and after 10 days in culture, cells appeared to have migrated down into the matrix (Fig. 3.1B) . The change in morphology was particularly apparent following growth on 1.25 and 2.5 mm gels and these progressively contracted after 2 days in culture. None of these features was affected by the presence of 1,25D.

3.4.2 Effect of matrix components on cell proliferation

Cell counts were slightly reduced following treatment with 1,25D (Fig. 3.2) but different matrices exerted a variety of effects on cell number. Proliferation on 0.05 and 0.5 µg/cm^2 fibronectin was not significantly different from that observed on tissue culture plastic, in the presence or absence of 1,25D. However, at concentrations of 5 and 50 µg/cm^2 in the absence of this inducing agent, proliferation was significantly increased (Fig. 3.2A). Laminin exerted no significant effect on cell proliferation in the presence or absence of 1,25D over a concentration range of 1-100 µg/cm^2 (Fig. 3.2B). In contrast, growth on collagen I film (Fig. 3.2C) and collagen I gel (Fig. 3.2D) reduced cell number at most ECM concentrations tested. This effect was observed irrespective of the presence or absence of 1,25D, but in the absence of this steroid, the inhibition of cell proliferation was more pronounced.

3.4.3 Effect of trypsin and collagenase on alkaline phosphatase activity

Alkaline phosphatase is a membrane bound enzyme which could potentially be degraded by enzymes such as trypsin and collagenase. Therefore preliminary experiments were undertaken to assess the effects of these enzymes on alkaline phosphatase activity (Methods 3.3.1 and 3.3.2). Results from these studies showed that there was no significant loss of alkaline phosphatase following treatment of human bone-derived cells with either trypsin or collagenase (Table 3.1).

Table 3.1A

	-Trypsin		+ Trypsin	
	+1,25D	-1,25D	+1,25D	-1,25D
µmol ρ-nitrophenol/ml/hr	0.544 (0.011)	0.198 (0.020)	0.590 (0.022)	0.215 (0.010)

Table 3.1B

	µmol ρ-nitrophenol/ml/hr	
Incubation with collagenase (min)	Control	+1,25D
0	0.440 (0.010)	0.874 (0.036)
30	0.405 (0.020)	0.904 (0.020)
60	0.432 (0.020)	0.850 (0.030)

Table 3.1. Effect of trypsin (Table 3.1A) and collagenase (Table 3.1B) on cellular alkaline phosphatase activity. Human bone-derived cells were cultured in the presence or absence of 1,25D. After 72 hr, cells were treated with trypsin or collagenase and cellular alkaline phosphatase activity was assessed. Values obtained for each treatment represent means and standard deviations from six wells each of which was assayed in triplicate.

3.4.4 Effect of matrix components on alkaline phosphatase activity when related to cell number.

Alkaline phosphatase activity was increased following addition of 1,25D irrespective of ECM applied (Fig. 3.3). Enzyme activities obtained following growth on 0.05 and 0.5 µg/cm^2 fibronectin were not significantly different from those observed on tissue culture plastic (Fig. 3.3A). However in the presence of 1,25D, at concentrations of 5 and 50 µg/cm^2, cellular alkaline phosphatase was significantly increased. Culture on collagen I film over a concentration range of 0.05-5 µg/cm^2 did not affect enzyme activity in the presence or absence of 1,25D. However, at a concentration of

50 μg/cm^2, in the presence of 1,25D, alkaline phosphatase activity was statistically reduced (Fig. 3.3B). Growth on collagen I gels caused a marked reduction in enzyme activity both in the presence and absence of 1,25D (Fig. 3.3C): on this matrix, cells expressed up to three fold less alkaline phosphatase than control cells cultured on tissue culture plastic.

3.4.5 Effect of matrix components on alkaline phosphatase activity when related to cellular DNA.

In all experiments, alkaline phosphatase activity was increased following treatment with 1,25D but basal and stimulated levels varied between experiments even when cells were cultured on tissue culture plastic (Fig. 3.4). This was thought to result from the fact that osteoblast-like cells were derived from patients of different age, sex and state of health. Culture on different concentrations of fibronectin had no consistent effect on alkaline phosphatase in the presence or absence of 1,25D (Fig. 3.4A). Similarly, culture on laminin at concentrations of 1 and 10 μg/cm^2 did not affect enzyme activity (Fig. 3.4B). However, at concentrations of 50 and 100 μg/cm^2 laminin, enzyme activity was reduced, in the presence and absence of 1,25D. Growth on collagen I film at a concentration of 0.05 μg/cm^2 did not affect enzyme activity (Fig. 3.4C). However, at concentrations of 0.5, 5 and 50 μg/cm^2, enzyme activity was reduced both in the presence and absence of inducing agents. Growth on collagen I gel also caused a marked decrease in alkaline phosphatase activity (Fig. 3.4D): this effect was independent of the depth of gel and more pronounced in the presence of 1,25D.

3.4.6 Effect of matrix components on osteocalcin production

Osteocalcin was only detected following treatment of bone-derived cells with 1,25D and the amount secreted decreased with increasing concentration of matrix applied (Fig. 3.5) . The only exception to this occurred following growth on 50 μg/cm^2 laminin which reproducibly increased osteocalcin towards levels observed on tissue culture plastic (Fig. 3.5B). The general dose dependent decrease in osteocalcin release was particularly pronounced following growth on collagen I gels (Fig. 3.5D) and suggested possible retention by the matrix. Preliminary experiments (Method 3.3.3) showed that trypsin and collagenase interfered with the osteocalcin RIA (Fig. 3.6A). However, dispersed collagen fibres did not affect the osteocalcin standard curve (Fig. 3.6B) and therefore osteocalcin released into collagen I gels was assessed following mechanical disruption. Although the levels obtained following growth on tissue culture plastic varied between experiments, total osteocalcin was progressively

reduced following growth on increasing depths of collagen I gel (Fig. 3.7). This showed that the decreased osteocalcin secretion into the culture medium (Fig. 3.5D) did not result from increased retention by the collagen matrix.

3.5 Discussion

This study compares the effects of culture on different matrix components on the expression of the osteoblastic phenotype in human bone-derived cells. The apparent similarity between morphology on tissue culture plastic and different concentrations of fibronectin, laminin and collagen I film can be explained by the fact that most cells adopt a characteristic flattened morphology when they adhere and proliferate on any solid substratum (Knox and Griffiths, 1980). However, laminin has been shown to elicit distinct morphological changes in other cell types. For example, Sertoli cells become more columnar, Schwann cells become more elongated and B16 melanoma cells form cell aggregates when cultured on this matrix (reviewed by Kleinman et al.1985). The change in cell shape observed following growth on collagen I gel correlates with data reviewed by Grinnell (1982). The fact that contact with collagen I film did not affect morphology suggests that the organisation of the collagen matrix and/or the ability of cells to penetrate this matrix are important factors in promoting changes in morphology. Gel contraction was typically observed following growth on 1.25 and 2.5 mm gels and interestingly, these wells produced the most dramatic changes in morphology.

Proliferation of human osteoblast-like cells varied with ECM applied. The increased cell number observed following growth on fibronectin in the absence of 1,25D correlates with other data obtained using embryonic and tumour cell lines (reviewed by von der Mark et al.1992). However, the absence of any proliferative effects following growth on different concentrations of laminin does not agree with other studies performed in non bone cell types which demonstrate a growth promoting effect of this ECM protein (reviewed by Kleinman et al.1985). Conflicting results have been obtained for the effects of laminin on the clonal osteoblast-cell line MC3T3-E1: Vukicevic et al.(1990) showed a time dependent stimulation of proliferation over a period of 8 days whereas Schotland et al.(1992) demonstrated a 60% reduction in cell proliferation over 1-15 days. The anti-proliferative effects of collagen I film and collagen I gel appear to be more consistent and the results of these studies are in good agreement with those of Vukicevic et al.(1990) and Schotland et al.(1992). Throughout these studies, 1,25D slightly reduced cell number, a phenomenon which

typically accompanies induction of a more differentiated osteoblast phenotype in this culture system (Beresford et al.1984b; Skjodt et al.1985; Beresford et al.1986).

The induction of alkaline phosphatase activity observed following addition of 1,25D is a well documented response of human osteoblast-like cells and is used for their characterisation (Beresford et al.1986). Growth on fibronectin did not generally affect basal or stimulated levels of alkaline phosphatase: although enzyme activity was slightly induced in one experiment when results were related to cell number, no such response was observed in three subsequent experiments when results were expressed per μg DNA. In contrast, growth on increasing concentrations of laminin reduced alkaline phosphatase activity particularly in the presence of 1,25D. These results do not correlate with findings observed by Schotland et al.(1992) who demonstrated an increase in alkaline phosphatase after culturing MC3T3-E1 cells on this matrix. Experiments performed in other cell types also suggest that laminin in some way promotes differentiation. For example, laminin has been shown to stimulate differentiation of embryonic F9 cells, teratocarcinoma cells and B16 melanoma cells in different culture systems (reviewed by Kleinman et al.1985). The decreased levels of alkaline phosphatase activity observed following growth of human osteoblast-like cells on collagen I film and collagen I gel correlates with studies performed by Schotland et al.(1992), who demonstrated a 50% reduction in alkaline phosphatase after culturing MC3T3-E1 cells on collagen I film. However, when bone cells isolated from rat calvariae were seeded onto collagen I gel, a 127% increase in alkaline phosphatase activity was observed when compared to levels obtained on tissue culture plastic (Guerra et al.1992).

The absence of any detectable osteocalcin under basal conditions and its induction by 1,25D is another characteristic feature of human bone-derived cells (Beresford et al.1984b). In these experiments, growth of human osteoblast-like cells on increasing concentrations of fibronectin, collagen I film and laminin and on different depths of collagen I gel typically decreased 1,25D induced osteocalcin release. This apparent reduction may have resulted from retention by the matrix through non-specific binding of matrix components or through induced cell polarisation and specific secretion at the basal surface. However, when total osteocalcin released by cells grown on collagen I gels was assessed, levels continued to decrease with increasing depth of collagen I gel. It is possible that the reduction observed following growth on collagen I gel resulted from altered availability of 1,25D. Collagen I gels contained large amounts of serum (~37 %) which could bind this low molecular weight compounds and lower its effective concentration. However, this reduction cannot be attributed purely to relative

availability since a decrease in osteocalcin release was also observed following growth on collagen I film. The progressive reduction in osteocalcin release with increasing depths of collagen I gel might also result from the availability of nutrients and oxygen. This would correlate with the marked reduction in proliferation following growth on 5 mm gels. However, the majority of cells remained towards the top of the matrix and moreover, no such effect was observed on alkaline phosphatase activity .

From these experiments, it can be seen that different matrix components exerted a variety of effects on human bone cell phenotype. Growth on different concentrations of fibronectin did not significantly affect cell morphology or alkaline phosphatase activity but it slightly increased proliferation and decreased 1,25D induced osteocalcin release. The similarities observed between growth on fibronectin and on tissue culture plastic can be partly explained by the fact that fibronectin within the serum contributes to cell attachment when cells are seeded onto tissue culture plastic (~7.5 μg/ml). Therefore conditions in control and fibronectin coated wells would be very similar. However, pre-coated wells would contain considerably more fibronectin than non-coated wells suggesting a higher affinity binding between the cell and its ECM. This could potentially contribute to the cells 'well-being' and thus result in increased cell proliferation. The apparent reduction in osteocalcin detected in supernatants could be due to cell polarisation and specific secretion into the matrix. However, the fact that this phenomenon occurred following growth on all matrices, together with the general adhesiveness of this glycoprotein, suggests that the decrease resulted from non-specific binding to fibronectin.

Growth on different concentrations of laminin did not affect cell morphology or cell proliferation. However, results from alkaline phosphatase and osteocalcin assays suggest that laminin decreased expression of the differentiated osteoblast phenotype. Although laminin has been shown to promote differentiation in many other cell types, a 'de-differentiating' response could be more advantageous to osteoblasts *in vivo*. Angiogenesis is a prerequisite for bone formation and laminin is a major component of the endothelial cell basement membrane. During early phases of bone formation and fracture repair, osteoprogenitor cells and osteoblasts are in contact with the invading vascular system. During these early phases of bone formation, osteoblasts and osteoprogenitor cells must produce the necessary cell numbers for subsequent matrix synthesis. By promoting a less differentiated osteoblast phenotype, laminin could help to ensure that cells remain in a proliferative phase.

Growth of human osteoblast-like cells on different concentrations of collagen I film and on different depths of collagen I gel reduced proliferation, alkaline phosphatase activity and 1,25D induced osteocalcin release. The 'de-differentiating' effects of both these matrices were more pronounced than those obtained for laminin and cellular responses to collagen I gels were typically more dramatic than those of collagen I films. The main difference in phenotypic effects of collagen I film and collagen I gel concerned cell morphology. The fact that cells cultured on collagen I gel exhibited a change in shape which could be correlated with gel contraction suggests that these two responses are intimately associated events. Both of these matrices affected cell proliferation and both reduced cellular alkaline phosphatase activity and 1,25D induced osteocalcin release. This suggests that contact with collagen I is sufficient to elicit a cellular response and that organisation of collagen fibres is not important for these phenotypic effects. Therefore, in these experiments, cell morphology, cell proliferation and differentiation do not appear to be linked.

The apparent 'de-differentiating' effects of type I collagen do not agree with studies performed using other cell types such as mouse mammary epithelial cells (Emerman and Pitelka, 1977) (see above) and hepatocytes. When hepatocytes are seeded onto plastic they round up and die within three days. If, however, they are seeded onto a collagen I gel, they attach and remain morphologically identifiable as hepatocytes for periods of 6-7 days in culture. Furthermore, if these collagen I gels are released from the culture dish, expression of cytochrome P450 is maintained (Strom and Michalopoulos, 1982). There is also some evidence to suggest that culture on a 3D matrix of type I collagen promotes a differentiated phenotype in osteoblast-like cells. For example Casser-Bette et al.(1990) demonstrated that when MC3T3-E1 cells are cultured on a matrix of denatured type I collagen for 56 days, cells exhibited characteristics typical of a differentiated osteoblast phenotype. This includes synthesis of type I collagen, matrix mineralisation and strong expression of alkaline phosphatase and osteocalcin in the absence of 1,25D. However, these results were not compared with data obtained from cells cultured for a similar time period on tissue culture plastic or on other 3D matrices such as agarose or methyl cellulose. Therefore the ability of type I collagen to induce a differentiated osteoblast phenotype in this system remains to be established.

From the experiments performed in our laboratory, one could speculate that culture on collagen I is encouraging progression through the osteoblast lineage and that the down regulation of gene expression and decreased proliferation represents the formation of osteocytes and/or osteoblast lining cells. These two cell types are both end cells of the

osteoblast lineage; osteoblast lining cells are formed in metabolically quiescent areas at the bone surface and osteocytes are produced when osteoblasts become surrounded by collagen matrix. However, this seems rather unlikely in view of the results obtained using MC3T3-E1 cells after 56 days culture on a matrix of denatured type I collagen fibres (Casser-Bette et al.1990).

It would have been very exciting if growth on a collagen matrix had increased alkaline phosphatase activity and osteocalcin release because this would provide direct evidence to suggest that the ECM is responsible, at least in part, for promoting osteoblast maturation and differentiation. It is possible that a longer time course is required for any ECM to promote a more differentiated osteoblast phenotype. Alternatively, collagen alone may not be sufficient for induction of the differentiated osteoblast phenotype and different results would perhaps have been obtained if these experiments were repeated using a combination of different ECM proteins. In view of the importance of matrix proteins in the presentation of cytokines (see Introduction), it would also have been interesting to perform some experiments using a combination of ECM components and cytokines. However, it is possible that an added exogenous matrix cannot substitute for cellular matrix synthesis. Deposition of type I collagen by osteoblast-like cells could form an important scaffolding for the temporal and spatial localisation of noncollagenous bone matrix proteins which could in turn participate in the regulation of bone cell function. Evidence from *in vitro* studies suggests that culture of human osteoblast-like cells in the presence of sodium ascorbate, which is important for collagen synthesis, also increases cell proliferation and production of the bone matrix proteins BSP and osteocalcin (Jon Beresford, personal communication). This suggests that proliferation and synthesis of type I collagen and noncollagenous bone matrix proteins are all closely associated events.

Figure 3.1A

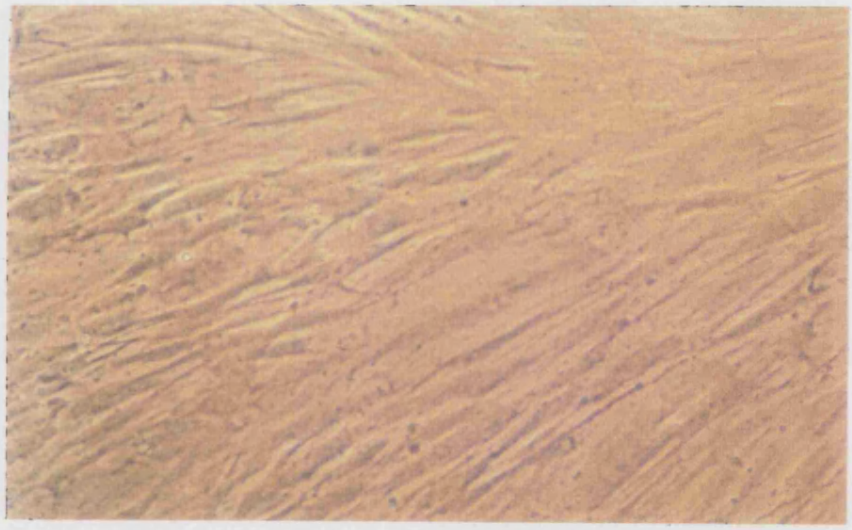


Figure 3.1B

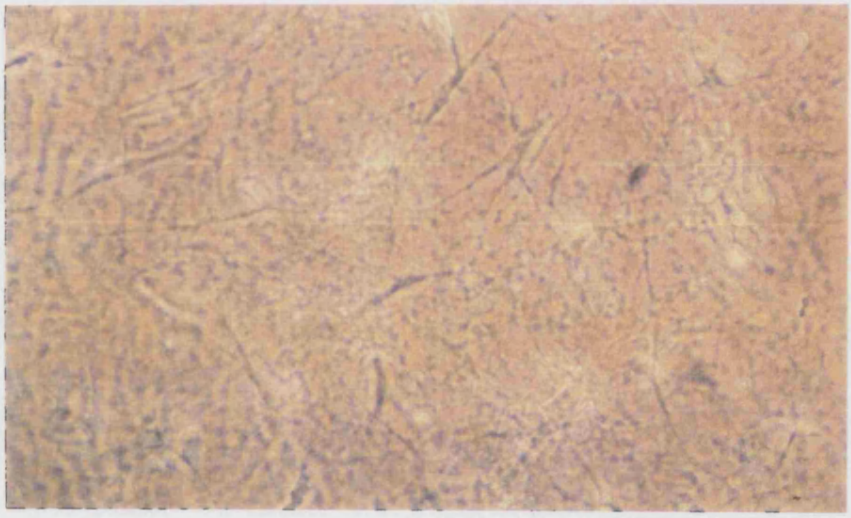
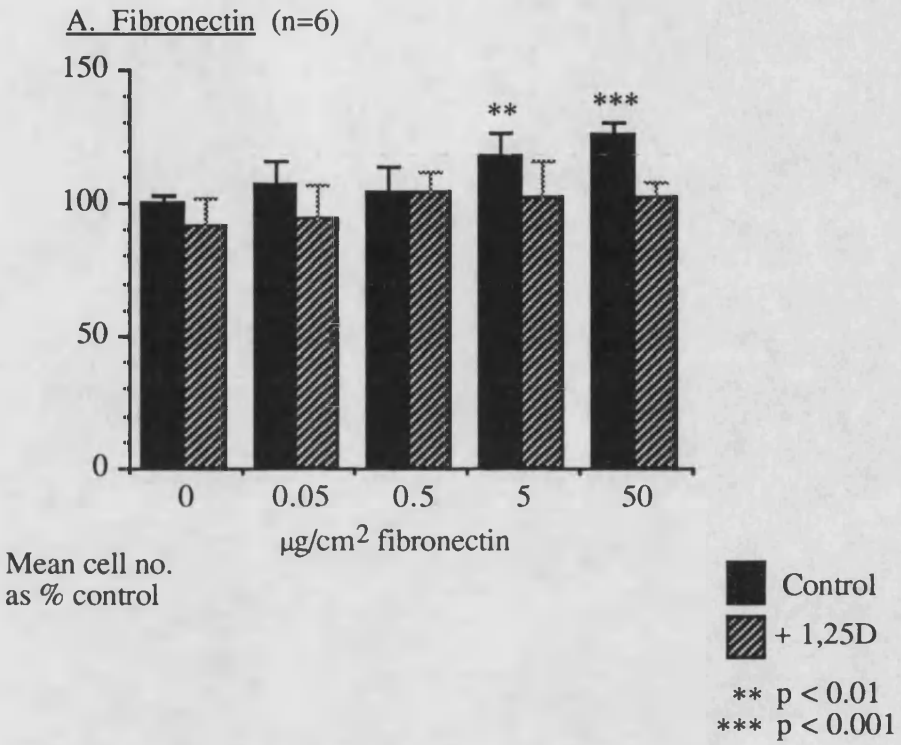


Figure 3.1 Morphology of human osteoblast-like cells following growth on tissue culture plastic (A) and collagen I gel (2.5 mm) (B).

After 10 days, cells grown on tissue culture plastic (Fig. 3.1A) exhibited a flattened, fibroblastic morphology. Cells cultured on a collagen I gel (Fig. 3.1B) displayed long cellular processes which penetrated the gel and after 10 days, cells appeared to have migrated down into the matrix. These changes in morphology were accompanied by gel contraction which typically occurred after 2 days in culture. None of these features were affected by the presence of 1,25D.

Figure 3.2



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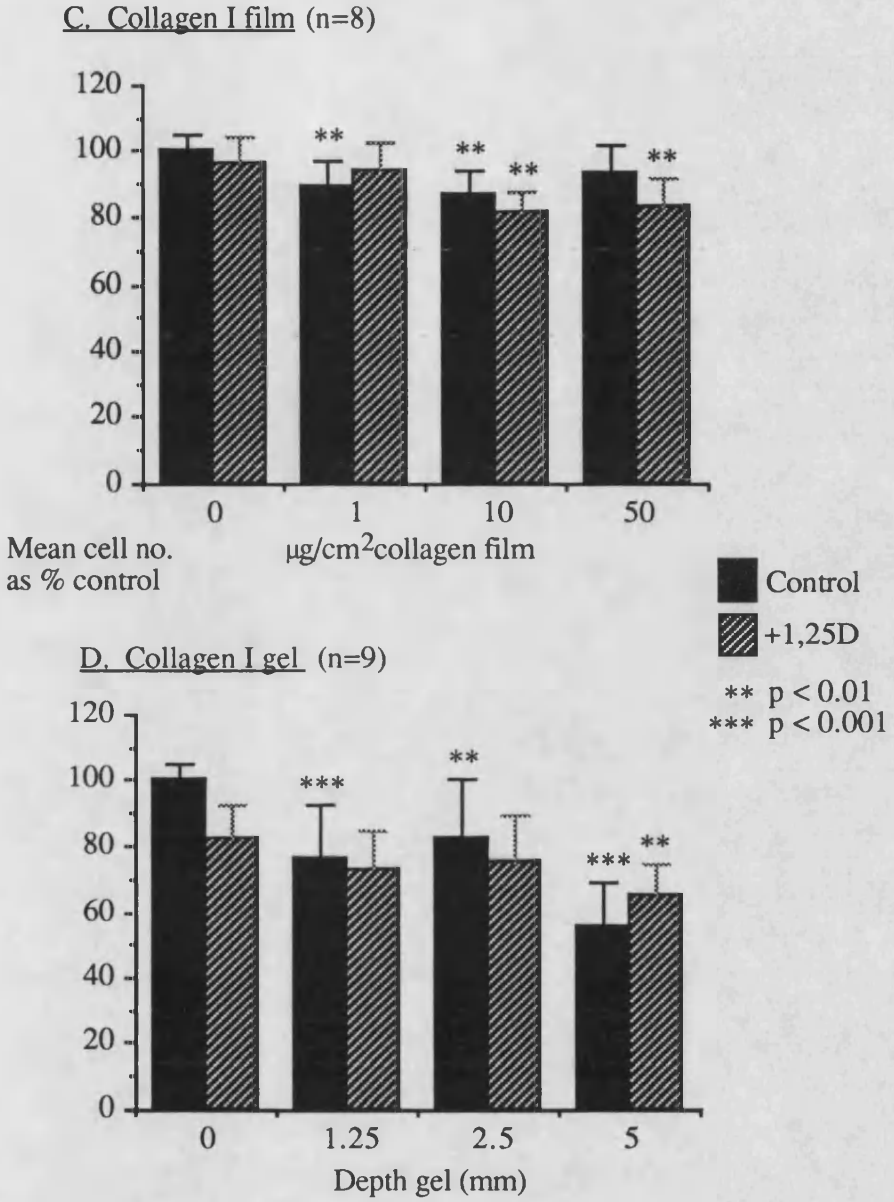
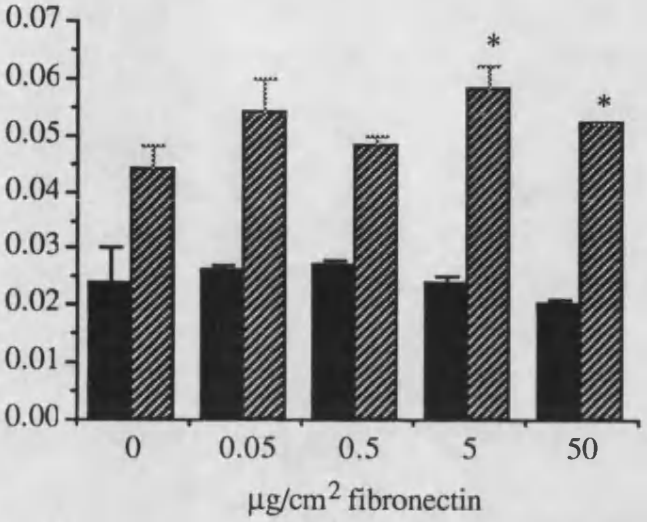


Figure 3.2 Effect of different matrix components on cell proliferation.

Human osteoblast-like cells were cultured on fibronectin (0-50 µg/cm2) (A), laminin (0-100 µg/cm2)(B), collagen I film (0-50 µg/cm2) (C) and collagen I gel (0-5 mm depth) (D); after 10 days, proliferation was assessed. Data were expressed as a % of control values i.e the cell counts obtained following growth on plastic in the absence of 1,25D (10^{-8}M). All histograms represent means and standard deviations from two or three different expts; in each expt two sets of counts were performed from 2 or 3 wells. All data were pooled and cell counts obtained following growth on different matrix components were compared with those observed on plastic +/- 1,25D. The significance of any change in cell number was determined using an unpaired Students T-test.

Figure 3.3

A. Fibronectin

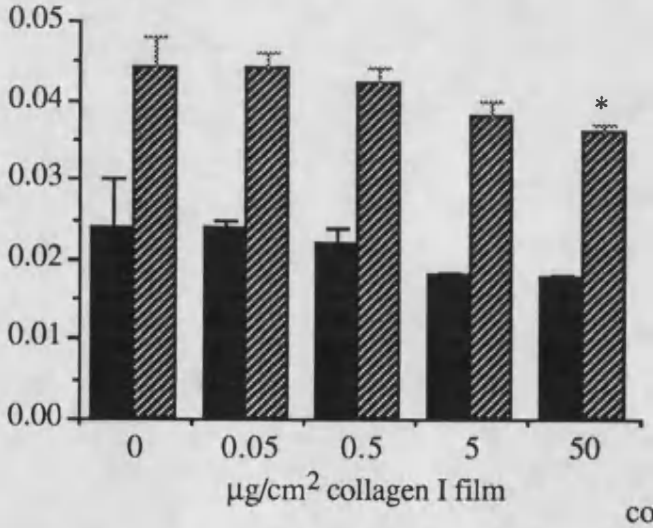


µmol
ρ-nitrophenol
/hr/10 000 cells

Control
+1,25D

* $p < 0.05$

B. Collagen I film



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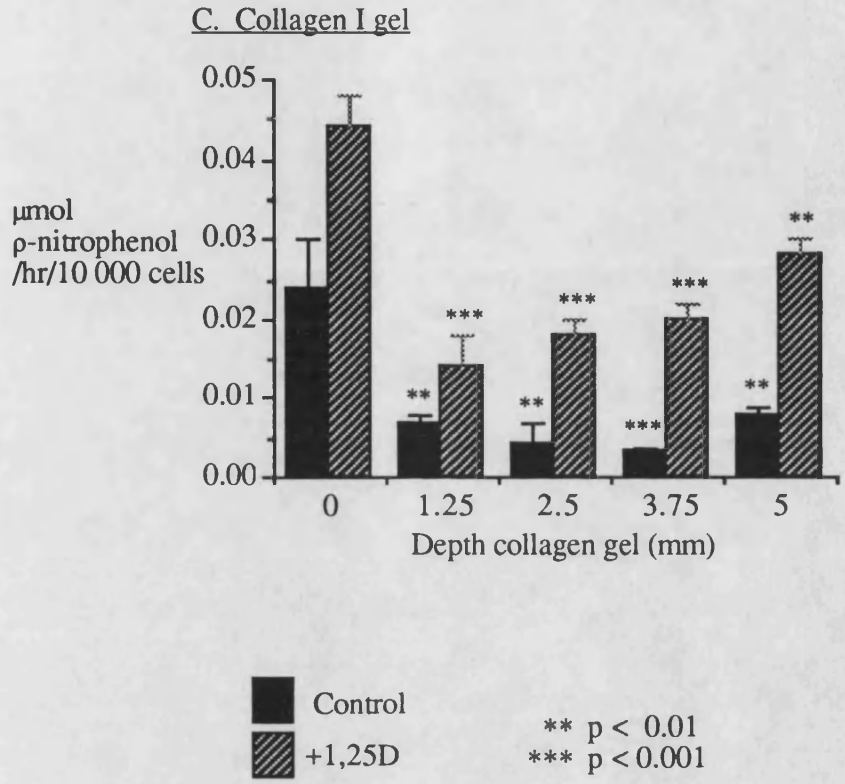
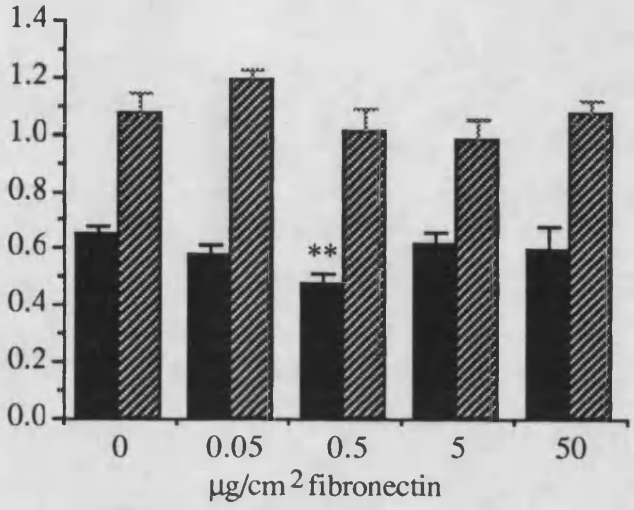


Figure 3.3. Effect of different matrix components on cellular alkaline phosphatase activity when related to cell no.

Human osteoblast-like cells were cultured on fibronectin (0-50 µg/cm^2) (A), collagen I film (0-50 µg/cm^2)(B) and collagen I gel (0-5 mm depth) (C). After 10 days, cells were detached and divided in a ratio of 1:2. Cells from the larger aliquot were assayed for alkaline phosphatase; cells from the smaller aliquot were counted in a haemocytometer. All absorbance readings were subtracted from blank values obtained by measuring ECM components digested in the absence of cells. Values represent means and standard deviations from three different wells each of each was assayed in triplicate. Enzyme activities obtained following growth on different matrix components were compared with results observed on plastic in the presence and absence of 1,25D (10^{-8}M). The significance of any change in enzyme activity was determined using an unpaired Student's T-test.

Figure 3.4

A. Fibronectin



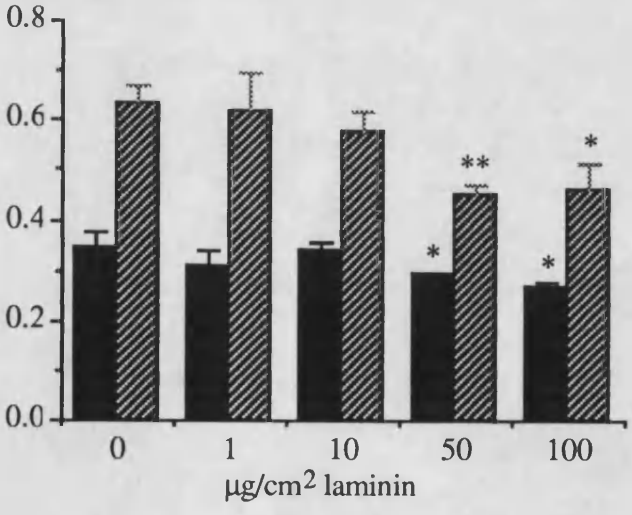
µmol ρ-nitrophenol /hr/µg DNA

Control

+1,25D

* p < 0.05
** p < 0.01

B. Laminin



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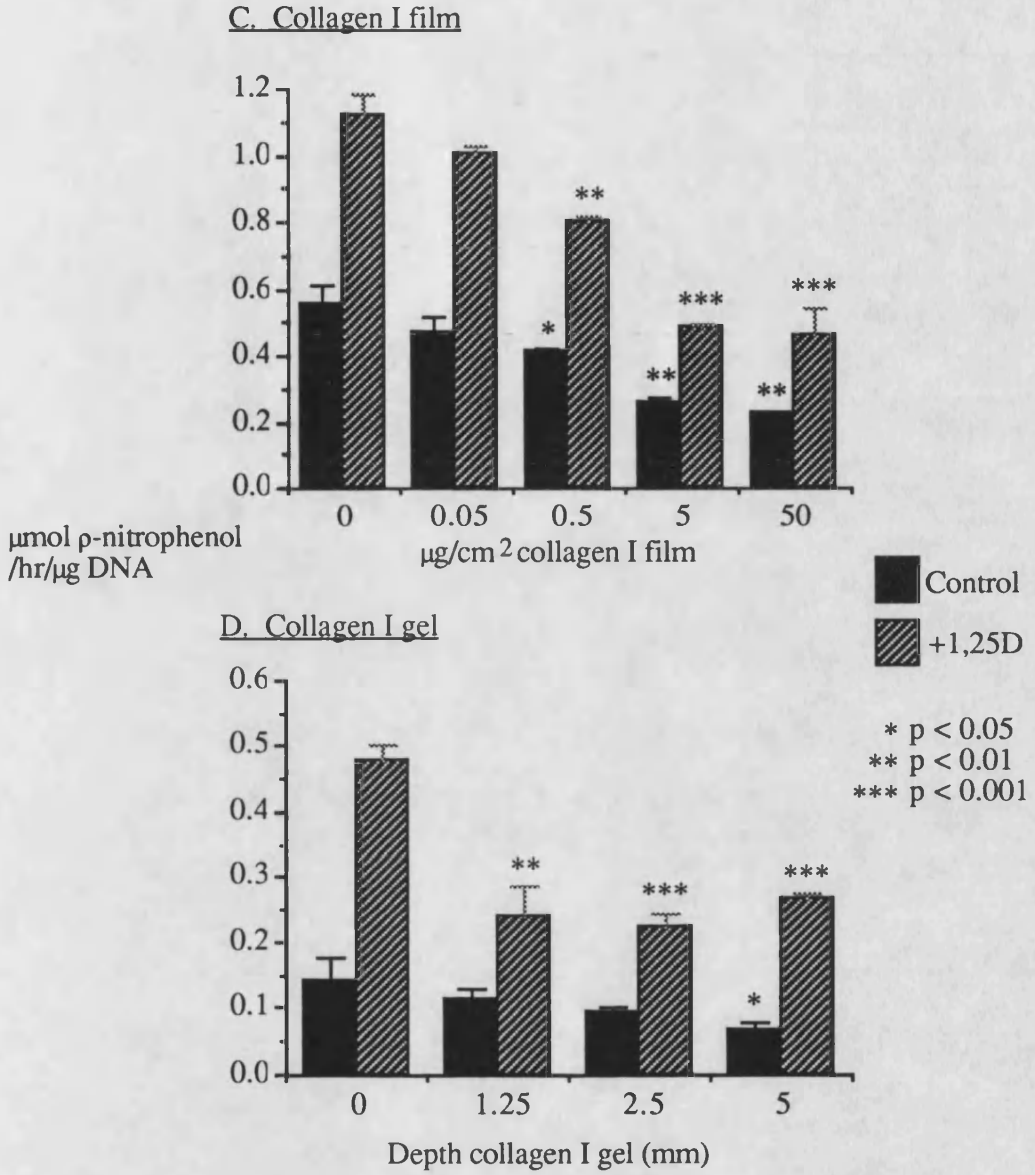
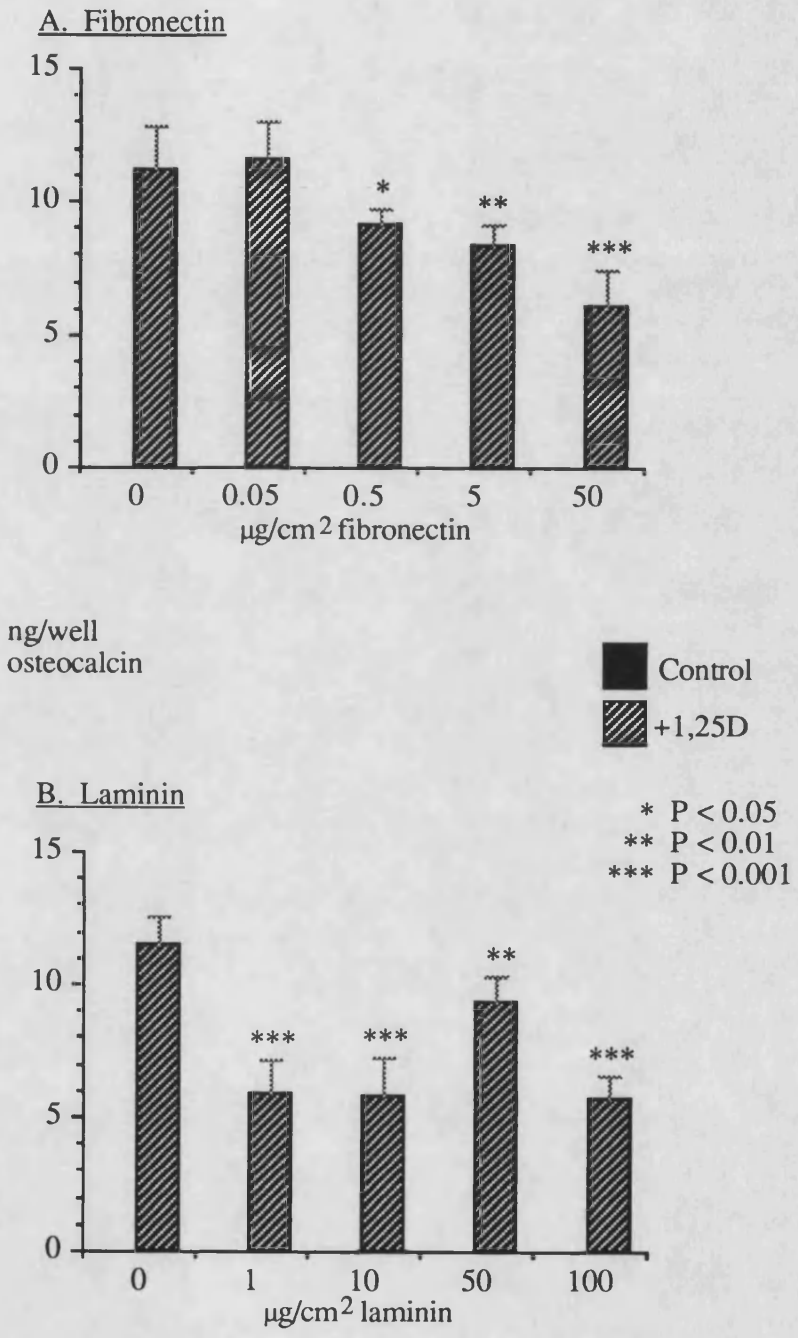


Figure 3.4 Effect of different matrix components on alkaline phosphatase activity when related to cell DNA.

Human osteoblast-like cells were cultured on fibronectin (0-50 μg/cm^2)(A), laminin (0-100 μg/cm^2)(B), collagen I film (0-50 μg/cm^2)(C), and collagen I gel (0.5 mm depth)(D). After 10 days, cells were detached and divided in a ratio of 1:2. Cells from the larger aliquot were assayed for alkaline phosphatase; cells from the smaller aliquot were assayed for cellular DNA. All absorbance and fluorescence readings were subtracted from blank values obtained by measuring ECM components digested in the absence of cells. Values represent means and standard deviations from three different wells each of which was assayed in triplicate. Enzyme activities obtained following growth on different matrix components were compared with results observed on plastic in the presence and absence of 1,25D (10^{-8}M). The significance of any change in enzyme activity was determined using an unpaired Student's T-test. Results are representative of three different experiments.

Figure 3.5



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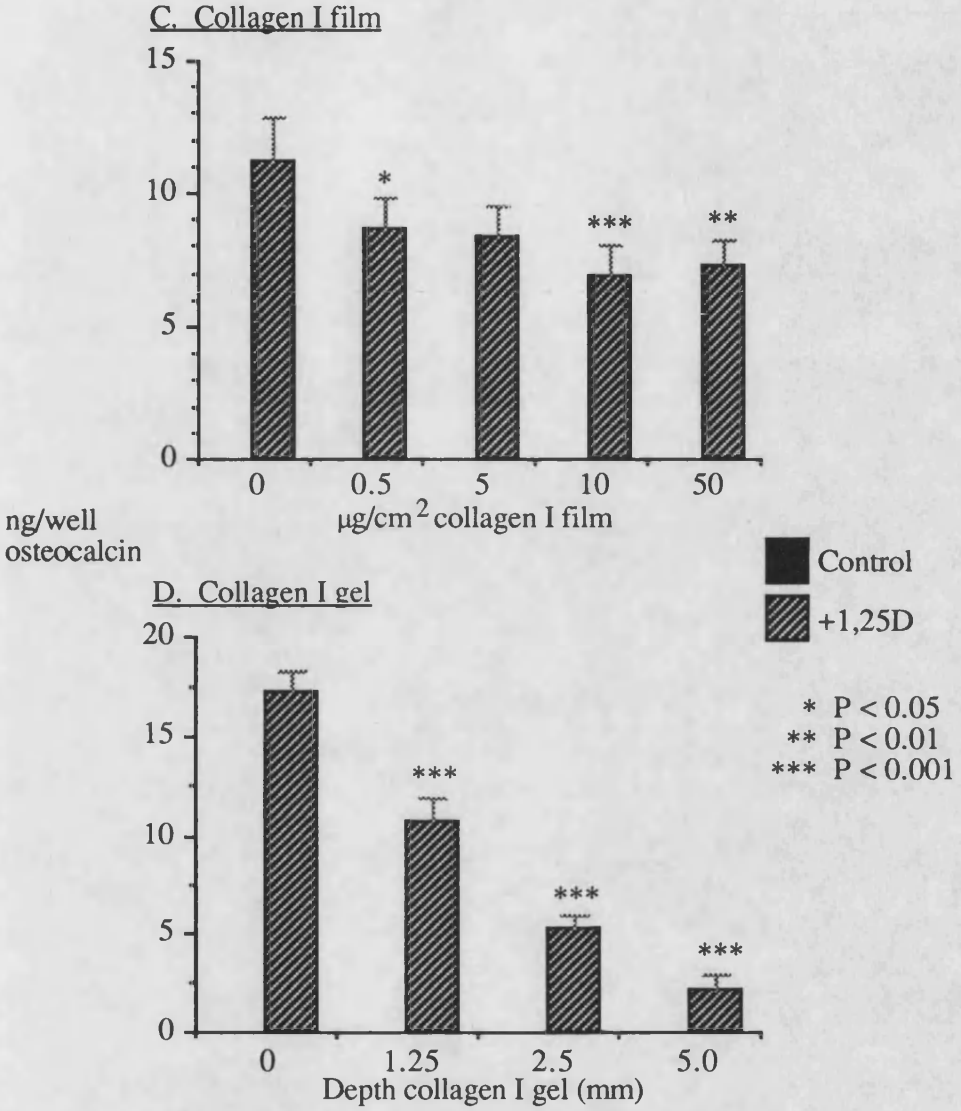
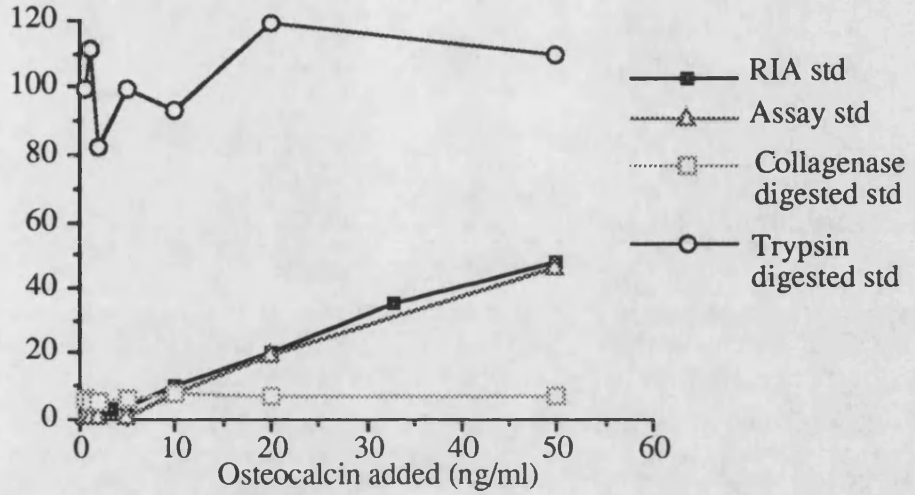


Figure 3.5. Effect of different matrix components on osteocalcin production.

Human osteoblast-like cells were cultured on fibronectin (0-50 µg/cm^2) (A), laminin (0-100 µg/cm^2) (B), collagen I film (0-50 µg/cm^2) (C), and collagen I gel (0-5 mm depth) (D). After 10 days, supernatants were assayed for osteocalcin production. All data were subtracted from blank values obtained for medium from wells coated with ECM proteins in the absence of cells. Values represent means and standard deviations from three different wells each of which was assayed in triplicate. Osteocalcin released by cells grown on different matrix components was compared with results observed on plastic in the presence and absence of 1,25D (10^{-8} M). The significance of any change in osteocalcin release was determined using an unpaired Student's T-test. Results are representative of three different experiments.

Figure 3.6A



Osteocalcin detected by RIA (ng/ml)

Figure 3.6B

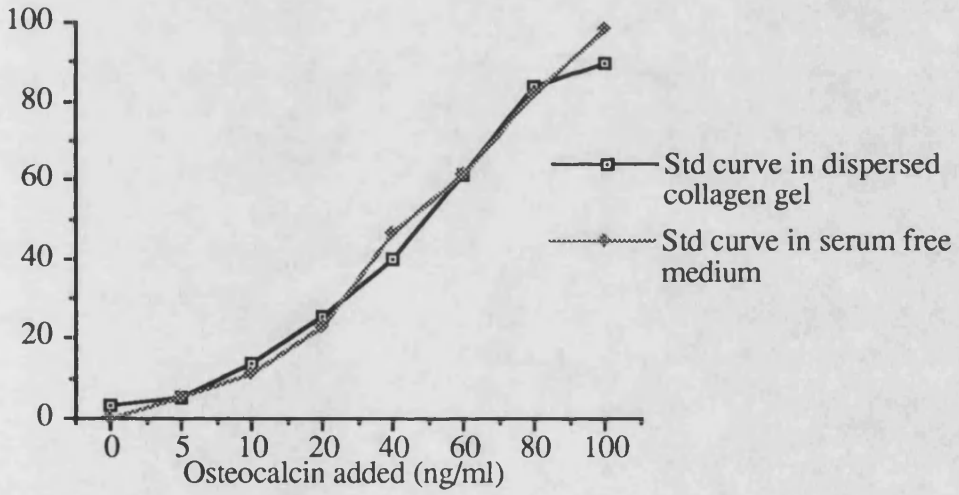


Figure 3.6. Effect of trypsin, collagenase (Fig 3.6A) and collagen fibres (Fig 3.6B) on the osteocalcin assay.

Standard curves were prepared in PBS from bovine osteocalcin and if necessary, overlayed with a collagen gel. Osteocalcin standards were quantified by RIA after treatment with trypsin or collagenase (Fig 3.6A) or in the presence of dispersed collagen fibres (Fig 3.6B). Data represent mean values obtained from two wells each of which was assayed in triplicate.

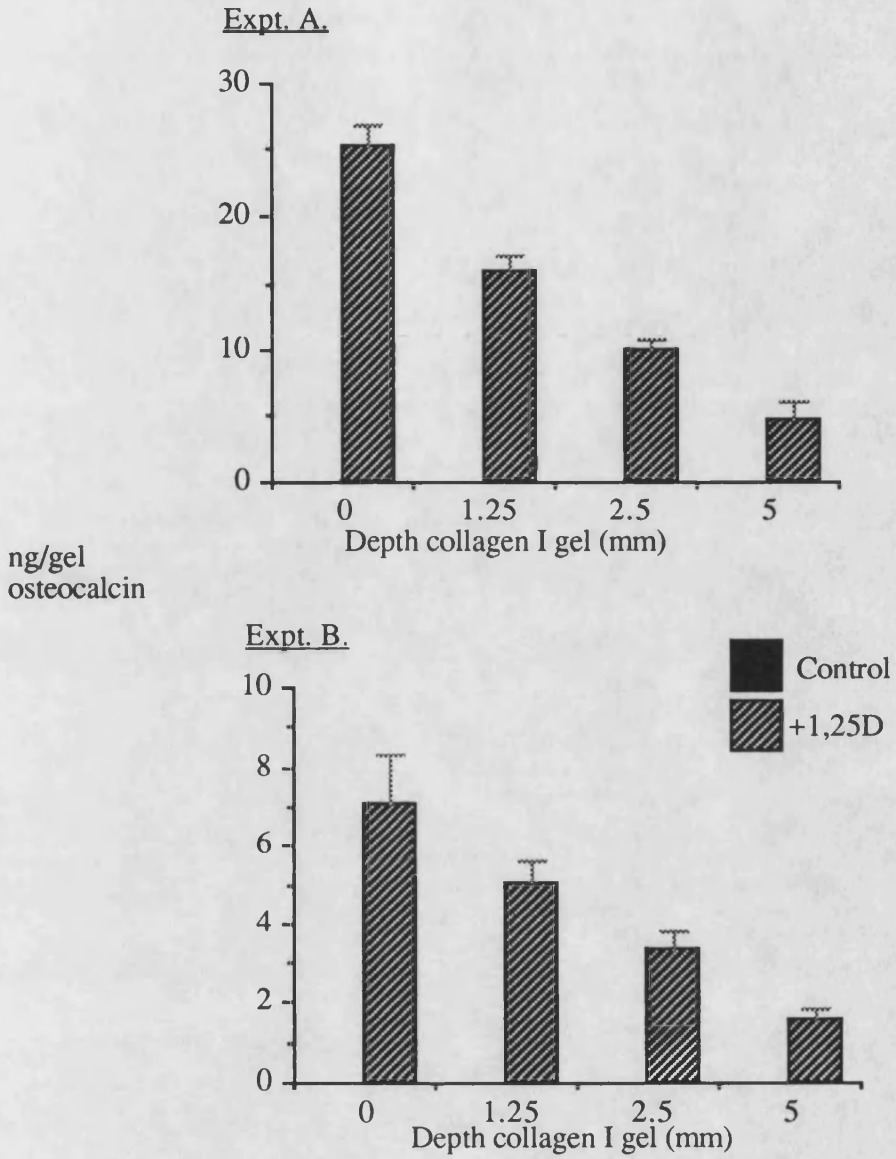


Figure 3.7. Total osteocalcin released by cells grown on plastic and collagen gels.

Human osteoblast-like cells were cultured on plastic or collagen gels (1.25-5 mm) for 10 days. At the end of the treatment period supernatants, dispersed collagen gels and scraped cells (from uncoated wells) were assayed for osteocalcin production. All data were subtracted from appropriate blank values and represent means and standard deviations from three different wells, each of which was assayed in triplicate.

CHAPTER 4.

INTEGRIN SUBUNIT EXPRESSION BY HUMAN OSTEOBLASTS AND OSTEOCLASTS *IN SITU* AND IN CULTURE

4.1 Abstract

The aim of this study was to establish which integrin subunits are present on human bone cells *in situ* and in culture, using cryostat sections of undecalcified human bone, osteoclastoma tissue and cultured human osteoblast-like cells. Integrin expression was identified indirectly using alkaline phosphatase anti-alkaline phosphatase conjugates and FITC-labelled secondary antibodies.

Staining patterns observed *in situ* show that osteoblasts and osteoclasts possess different integrin subunits. Osteoblasts express primarily α1, α3 and β1 and weakly express α2. Osteoclasts express α2, αV, β1 and β3. Subunits α4, α5, α6, αL, αM and β2 were not expressed by either of these cell types. Expression of β1 by all cells of the osteoblastic lineage was constitutive, but α1 and α3 subunits were expressed by osteoblasts actively synthesizing bone and some of the osteoblast lining cells. All integrin subunits identified on osteoblasts *in situ* were maintained on culture but there was an increased expression α2, and αV subunits were weakly positive. Expression of α2, α3, αV and β1 subunits was independent of cell density but expression of α1 was much greater in confluent cultures. Integrin expression of α2, αV, β1 and β3 by osteoclasts did not vary according to osteoclast size, location or activation state. There was no evidence of subunit polarisation towards the bone surface exhibited by either osteoblasts or osteoclasts.

4.2 Introduction

The ECM plays an important role in regulating cell function. Cellular interactions with the ECM occur through specific families of receptors, one of which are the integrins. These transmembrane proteins are αβ heterodimers which play a structural role in mediating adhesion, and in addition, transmit information between the outside and the inside of the cell. The cell surface expression of integrin subunits partly determines the local environment immediately surrounding the cell and cytoskeletal associations potentially affect the way in which a cell responds to signals from its environment.

In view of the importance of integrin molecules, a study was undertaken to establish the pattern of integrin subunits expressed by human bone cells *in situ* and in culture. Immunolocalisation experiments were performed using cryostat sections of undecalcified human osteophyte, osteoclastoma tissue and cultured human osteoblast-like cells. The sections were cut from developing osteophytes obtained from osteoarthritic femoral heads, or from osteoclastoma neoplasms (giant cell tumours); cells were cultured from explants of trabecular bone. Human osteophyte is highly cellular and extremely metabolically active and therefore provides a very good model for studying bone cells *in situ*. Osteoclastoma tissue was used because it is rich in the relatively rare, multi-nucleated osteoclasts (Horton et al.1985b). All immunolocalisation experiments were repeated using cultures of human osteoblast-like cells in order to determine whether integrin subunit expression was maintained during *in vitro* conditions. Integrin subunit expression was detected using mAbs and identified indirectly using alkaline phosphatase anti-alkaline phosphatase conjugates (APAAP) or FITC-conjugated secondary antibodies (Method 2.5).

4.3 Results

4.3.1 Histology of the osteophyte

Typical histological staining patterns observed for sections of human osteophyte are shown in Figure 4.1. This tissue provides a rich source of osteoblasts and osteoclasts and different stages of bone remodelling can be easily identified within the same section. For example, in this figure, metabolically quiescent areas, characterised by a continuous layer of osteoblast lining cells are visualised on several bone surfaces. However, immediately opposite one of these quiescent areas, there is evidence of active bone formation. Here, the osteoblasts are cuboidal and actively synthesizing bone matrix (osteoid). In addition, within the same section, there is evidence of bone

resorption where osteoclasts are attached to the bone surface. These histological findings are in sharp contrast with those observed in sections of 'normal' bone: this tissue is typically relatively acellular and osteoclasts are a very rare occurrence (not shown).

4.3.2 Titration of mAbs for antigenic detection

Titration experiments were performed with all the anti-integrin mAbs to obtain the optimum concentrations required for antigenic detection. MAb dilutions used in subsequent immunolocalisation studies are summarised in Table 4.1. Many of these mAbs were positive at much lower concentrations than those described, but it was decided to use all immunochemicals at saturating concentrations. Some mAbs were negative at all concentrations tested: in these situations, the functionality of the mAb was assessed using an appropriate positive control.

Table 4.1. MAb dilutions used for immunolocalisation experiments

Integrin subunit detected	Monoclonal antibody	Optimum dilution in sections of osteophyte	Detection in other cell types
α1	T52/7	1/50	
α2	G19	1/50	
α3	PB15	1/20	
α4	HP2/1	negative	lymphocytes, 1/50
α5	SAM1	negative	U937 cells, 1/20
α6	GoH3	negative	platelets; 1/50; sections of skin,1/100; blood vessels 1/100
β1	4B4	1/100	
αL	IOT16	1/100	
αM	R841	1/100	
β2	IOT18	1/100	
αV	23C6	neat	
β3	C22	neat	

Titration of anti-integrin mAbs in acetone fixed human osteoblast-like cells. MAbs derived from ascites were diluted between 1/25 and 1/3200 but mAbs from tissue culture supernatants were not diluted prior to use. All experiments were performed in the presence of appropriately diluted negative controls (IgG) and binding of mAbs was identified by APAAP or IF (Method 2.5).

4.3.3 Integrin subunit expression by bone cells *in situ* and in culture

Typical staining patterns observed from immunolocalisation studies are shown in Figs. 4.2-4.7 and summarised in Table 4.2. In studies employing the APAAP technique, positive areas stained red; any purple colouration resulted from the counterstain haematoxylin and represents mineralised bone and cell nuclei (Fig. 4.2).

The β1 subunit was strongly expressed by all osteoblasts in areas of recent/active bone formation (Fig. 4.3A), by all osteoblast lining cells (Fig. 4.3B) and also by osteocytes (Fig. 4.3C). In addition, positive staining was also apparent around the large multi-nucleated osteoclasts (Fig. 4.3C) and in the marrow (Fig. 4.3B). Expression of α1 and α3 subunits was similar: these subunits were expressed by all 'active' osteoblasts (Fig. 4.4A) and some osteoblast lining cells (Fig. 4.4B), but neither subunit could be detected on osteoclasts (Fig. 4.4C). The differential staining patterns obtained for cells of the osteoblastic lineage suggest that expression of some subunits varies according to cell phenotype. αV, β3 and α2 subunits were expressed by all osteoclasts (Fig. 4.5 and 4.6): expression of αV and β3 were similar (Fig. 4.5) but staining patterns observed for the α2 subunit were typically less intense than those observed for αV and β3 (Fig. 4.6A). Osteoblast lining cells were negative for all of these subunits but α2 was also occasionally visualised on osteoblasts in areas of active/recent bone formation (Fig. 4.6B). Integrin expression of α2, β1, αV and β3 by osteoclasts agrees with previous findings (Horton et al.1985a; 1985b; Horton and Davies, 1989) and did not vary according to size, location or activation state of the cells. Neither osteoblasts nor osteoclasts showed any evidence of polarisation towards the bone surface.

Subunits αL, αM, β2, α4, α5 and α6 could not be identified on either osteoblasts or osteoclasts. αL, αM and β2 subunits showed a similar pattern of expression which was restricted to the marrow (Fig. 4.7C): no staining was observed on bone surfaces (Fig. 4.7A) or on osteoclasts (Fig. 4.7B). α6 subunits could be visualised on endothelial cells lining the occasional blood vessel but all other cell types within osteophytic tissue were negative (data not shown). No staining was apparent with mAbs directed against α4 and α5 subunits (data not shown). This did not result from impaired functionality as α4 subunits could be identified on freshly prepared lymphocytes and α5 subunits were observed on U937 cells (a myeloid cell line) (see Table 4.1).

Table 4.2 Immunolocalisation of integrin subunits in cryostat sections of undecalcified human bone and in cultured osteoblasts

Integrin subunit detected	Monoclonal antibody	Reactivity in sections of osteophyte	Reactivity in cultured osteoblasts
α1 (CDw49a)	T52/7	Osteoblasts ++ Osteoclasts - Marrow +	++ (confluent cultures)
α2 (CDw49b)	G19	Osteoblasts +/- Osteoclasts ++ Marrow +	+
α3 (CDw49c)	PB15	Osteoblasts +++ Osteoclasts - Marrow +	++
α4 (CDw49d)	HP2/1	-	-
α5 (CDw49e)	SAM1	-	-
α6 (CDw49f)	GoH3	-	-
β1 (CD29)	4B4	Osteoblasts +++ Osteoclasts +++ Marrow +++	+++
αL (CD11a) αM (CD11b) β2 (CD18)	IOT16 R841 IOT18	Osteoblasts - Osteoclasts - Marrow ++	-
αV (CD51)	23C6	Osteoblasts - Osteoclasts +++ Marrow -	+/-
β3 (CD61)	C22	Osteoblasts - Osteoclasts +++ Marrow -	-

-, negative; +/-, 1% positive; +, positive; ++, more positive; +++, strongly positive

In situ staining patterns observed for cells of the osteoblastic lineage were maintained in culture (Figs. 4.3D, 4.4E, 4.4F) but there appeared to be increased expression of α2 (Fig. 4.6C), and αV subunits were weakly positive (data not shown). Expression of α2, α3, αV and β1 subunits was independent of cell density but expression of α1 was much greater in confluent cultures.

4.4 Discussion

In addition to its obvious structural role, the extracellular matrix of bone is concerned with the phenotypic regulation of associated cells. Therefore, the expression of selective receptors by these cells for specific ligands within the extracellular matrix is likely to be critical in controlling cell function.

Staining patterns obtained *in situ* showed that osteoblasts primarily express integrin subunits α1, α3 and β1. Both of these α subunits can combine with β1 forming α1β1 (VLA-1), a receptor for collagen and laminin, and α3β1 (VLA-3), a receptor for collagen, fibronectin and laminin. All staining patterns observed *in situ* were maintained in culture but there appeared to be increased expression of α2, and αV subunits became weakly positive.

Osteoclasts expressed a different selection of integrin subunits, namely α2, αV, β1 and β3; findings which correlate with those reported previously (Horton et al.1985a; 1985b; Horton and Davies, 1989). β1 can combine with α2 forming a receptor for collagen and laminin, or with αV to form a receptor which binds a range of ligands such as collagen, fibronectin, vitronectin, fibrinogen and von Willebrand's factor (Humphries, 1990). αV can also associate with β3 to encode a receptor with a broad specificity: possible ligands include fibronectin, vitronectin, laminin, osteopontin, bone sialoprotein, thrombospondin, fibrinogen and von Willebrand's factor. Osteopontin has been localised in the bone matrix underlying resorbing rat osteoclasts which may indicate a possible involvement of this protein in osteoclast adhesion *in vivo* (Mark et al.1987; Reinholt et al.1990). In the more recent of these studies (Reinholt et al.1990), high levels of osteopontin were localised in the clear zone of the osteoclast plasma membrane whereas much less staining was observed in the ruffled border and deeper within the bone. Highest expression of the vitronectin receptor (αVβ3) was also observed at the clear zone, suggesting that osteopontin may function as an anchor of osteoclasts to bone by binding to the vitronectin receptor and to hydroxyapatite. However, in another study, αVβ3 was not shown to mediate tight sealing zone attachment of osteoclasts to the bone surface (Lakkakorpi et al.1991). Therefore the

receptor-ligand interactions required for attachment of osteoclasts to the bone matrix are yet to be established.

Niether osteoblasts or osteoclasts expressed αL, αM and β2 subunits but positive staining was observed in the marrow. This staining pattern is to be expected as expression of these subunits is restricted to leukocytes (Humphries, 1990). The result obtained for osteoclasts is interesting because leukocytes and osteoclasts are thought to be derived from a common pluripotent stem cell (CFU-GM). Negative osteoclastic staining could suggest that expression of these antigens is lost during the fusion of mononucleated osteoclast precursor cells. Alternatively, this result could imply that osteoclastic and leukoctyic lineages diverge at an ealy stage in their pathways of differentiation.

Subunits α4, α5 and α6 could not be detected on bone cells *in situ* or in culture. α4 can combine with β1 to form a fibronectin receptor (VLA-4) which recognises the alternatively spliced region of fibronectin. The negative findings correlate with previous reports suggesting that this subunit is expressed by resting lymphocytes, monocytes, neural-crest derived-cells (Springer, 1990) and melanoma cells (Albelda and Buck, 1990). Staining patterns obtained using monoclonal antibodies against α5 and α6 subunits were also negative: α5β1 (VLA-5) encodes a classical fibronectin receptor and α6 can associate with β1 to form a laminin receptor (VLA-6). The result obtained for α5 correlates with reports suggesting that this subunit is poorly expressed in most tissues (Albelda and Buck, 1990), but the negative finding observed for α6 is surprising as the laminin receptor is considered to be present on most cell types (Albelda and Buck, 1990).

Apart from mediating cell adhesion, the integrin molecules play an important role in transmitting signals between the external environment and the interior of the cell. As mentioned previously, one mechanism by which this communication could occur is through alterations in the cell surface expression of integrin subunits. This may be relevant in the context of the osteoblast in that the expression of α1, α2 and α3 subunits is restricted to certain osteoblast populations. However it is not possible to determine whether this is a cause or a consequence of the altered phenotype. Individual cells can also vary their adhesive properties by regulating the specificity and affinity of a given integrin molecule (see Introduction). This may be important in the case of osteoclast αVβ3 receptor, which is constitutively expressed on osteoclasts irrespective of their site in bone.

The fact that osteoblasts and osteoclasts expressed a different pattern of integrin subunits suggests that these two cell types adhere to different ligands within the ECM and respond to signals from the external environment in different ways. Further studies to determine whether there is a difference in the adhesive specificity of these two cell types now need to be undertaken. The ligand binding ability of rat osteoclasts has already been investigated *in vitro* (Helfrich et al.1992). In this study osteoclasts adhered well to proteins such as osteopontin, BSP, vitronectin and fibronectin but failed to adhere to laminin. Attachment assays undertaken in our laboratory demonstrated that human osteoblast-like cells adhered well to laminin (data not shown), which suggests that the integrin subunit expression of osteoblasts and osteoclasts is indeed important for adhesive recognition. This could have important implications for the local control of bone remodelling where the amount of osteoclastic bone resorption at a given site is balanced by osteoblastic bone formation. It is possible that alterations in the composition of the ECM may favour the activity of one or other of these cell types. In support of this, there is evidence in a rat model that the ability of bone matrix to recruit osteoclasts decreases with age (Groessner-Schriber et al.1991). This could lead to an imbalance and might eventually result in pathological conditions such as osteoporosis.

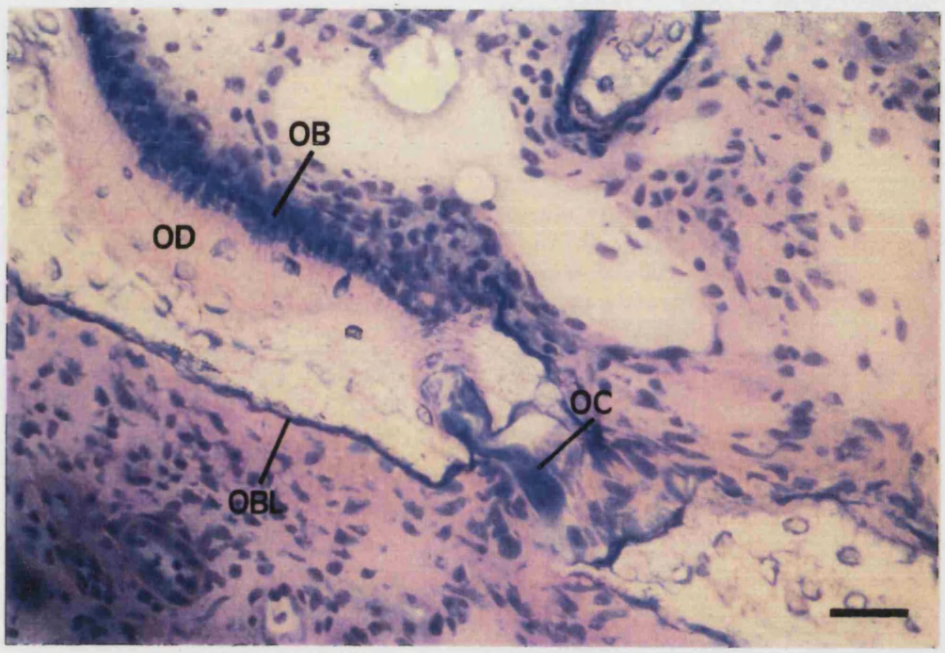


Figure 4.1 Histology of the osteophyte

Metabolically quiescent areas are characterised by a continuous layer of flattened osteoblast lining cells (OBL). In areas of active bone formation, cuboidal osteoblasts (OB) actively synthesise osteoid (OD). In areas of bone resorption, osteoclasts (OC) attach to the bone surface and breakdown bone matrix. Original magnification x 200. Bar, 45 μm

Figure 4.2

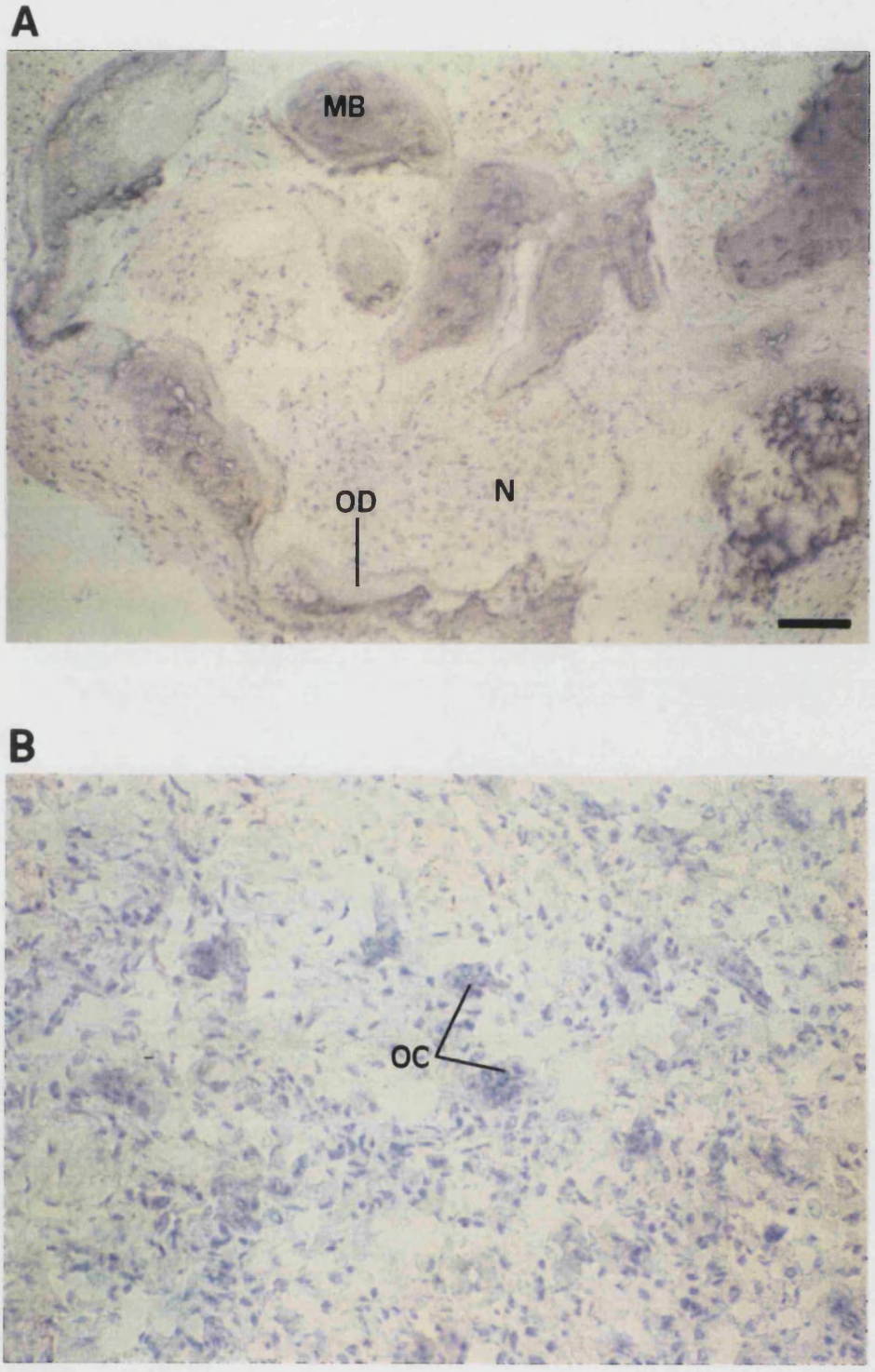


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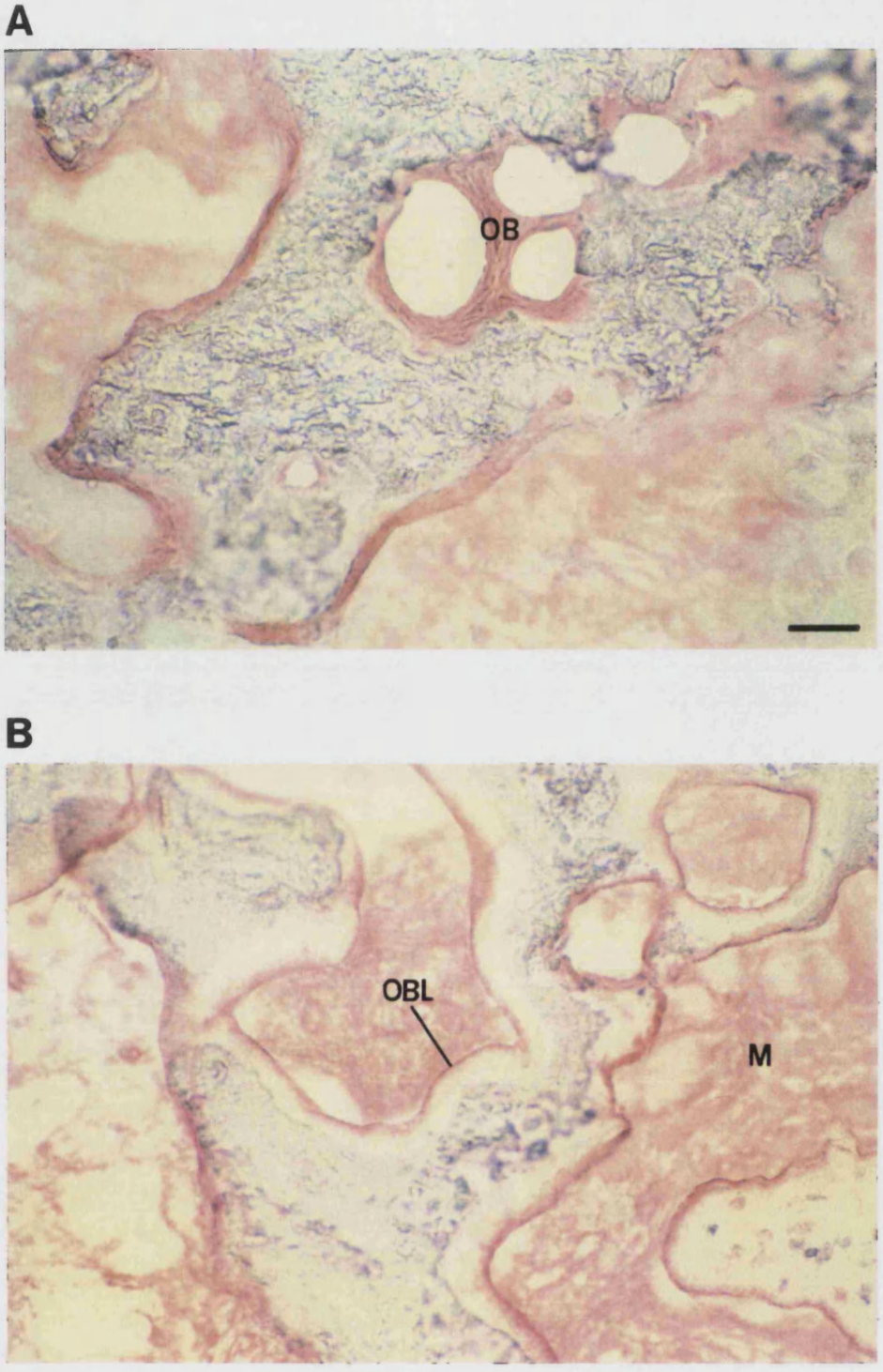
Figure 4.2 Negative control (mouse IgG) of integrin subunit expression in cryostat sections of undecalcified osteophyte, in sections of osteoclastoma and cultured osteoblast-like cells.

In sections of osteophyte (A), haematoxylin stains mineralised bone (MB) and cell nuclei (N): the lighter areas surrounding the mineralised bone represent newly formed non-mineralised osteoid (OD). In sections of osteoclastoma (B), haematoxylin stains cell nuclei thus enabling the large multi-nucleated osteoclasts (OC) to be easily visualised. In cultured osteoblast-like cells (C), haematoxylin stains cell nuclei (N). Original magnification: A x 100; B x 200; C x 100. Bar, 45 μm

Figure 4.3



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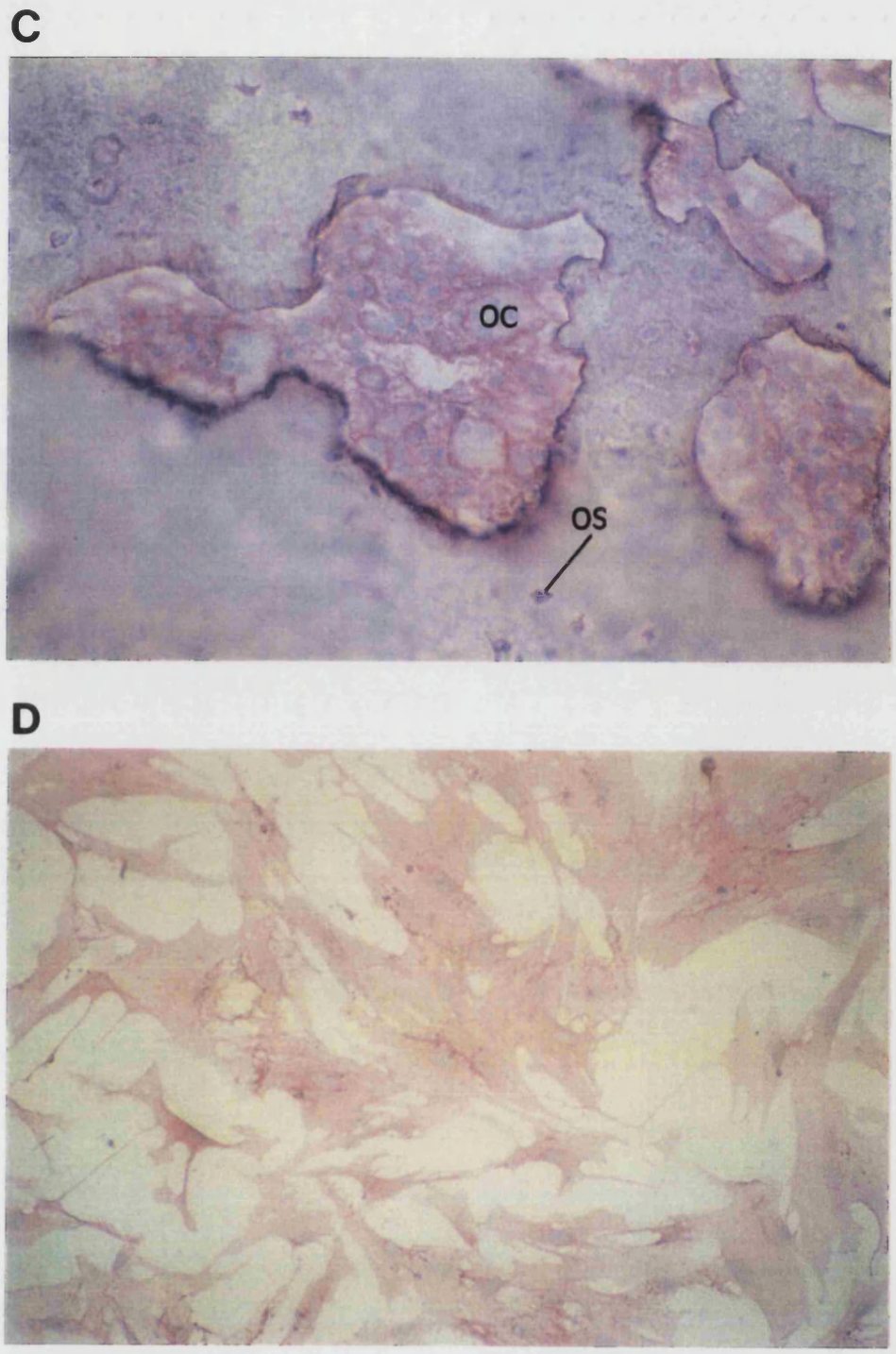
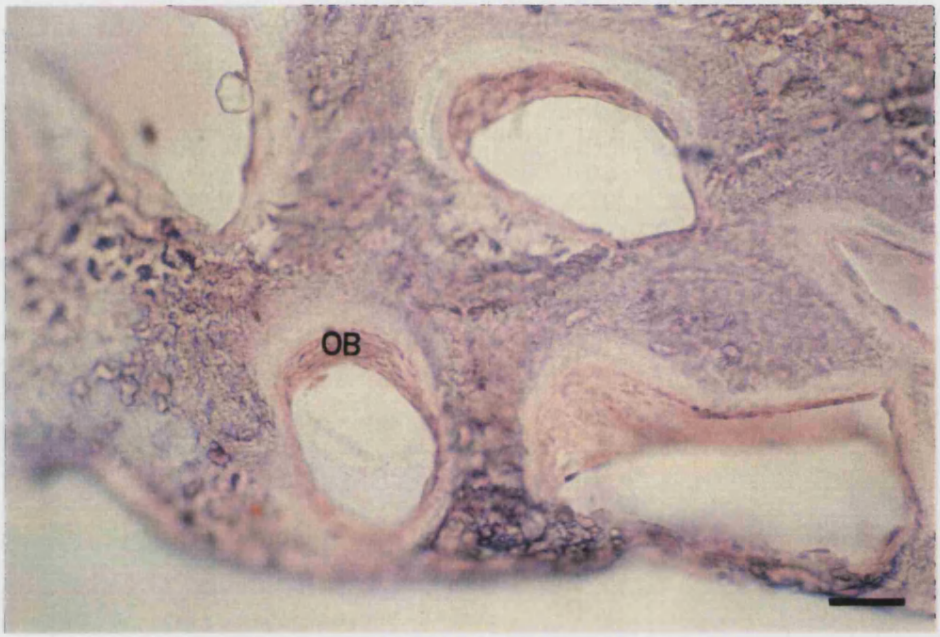


Figure 4.3 Expression of β1 subunits in cryostat sections of undecalcified osteophyte and cultured osteoblast-like cells.

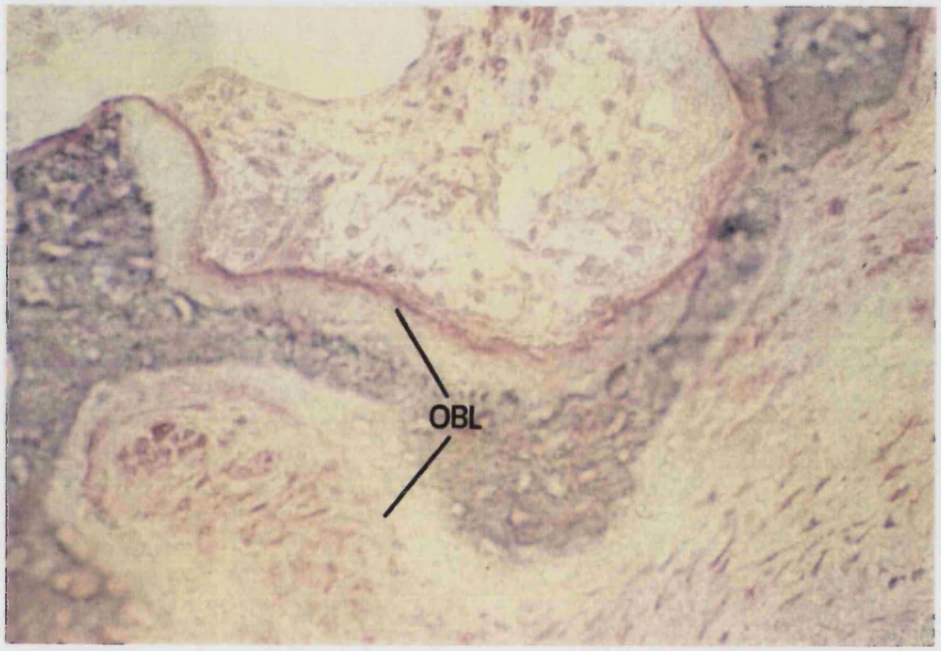
Osteoblasts (A; OB), osteoblast lining cells (B; OBL), marrow cells (B; M), osteocytes (C; OS) and osteoclasts (C; OC) are strongly positive. Expression by osteoblast-like cells is maintained in culture (D).
Original magnification: A, B, C, D x 200.
Bar, 45 μm

Figure 4.4

A



B



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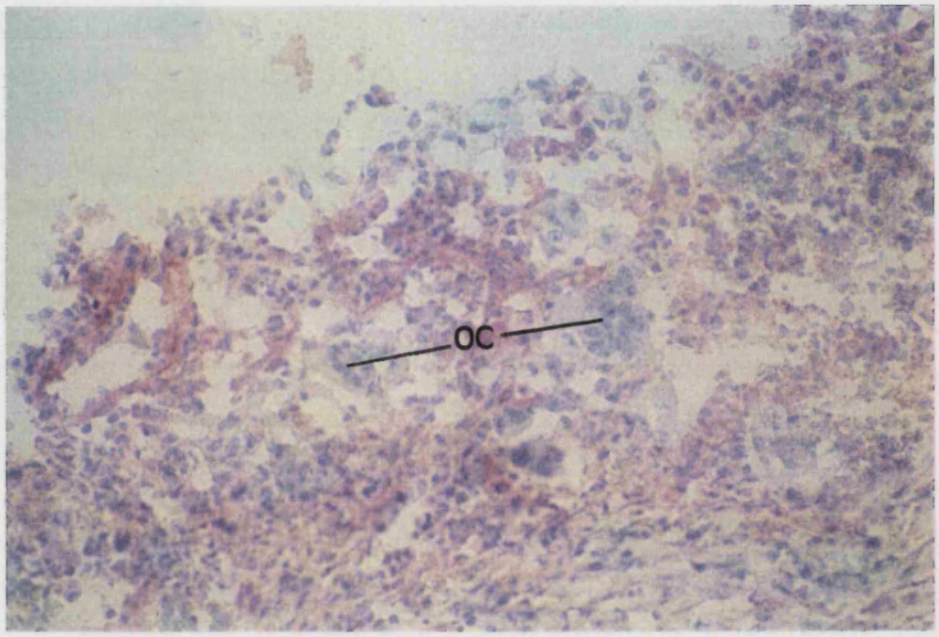
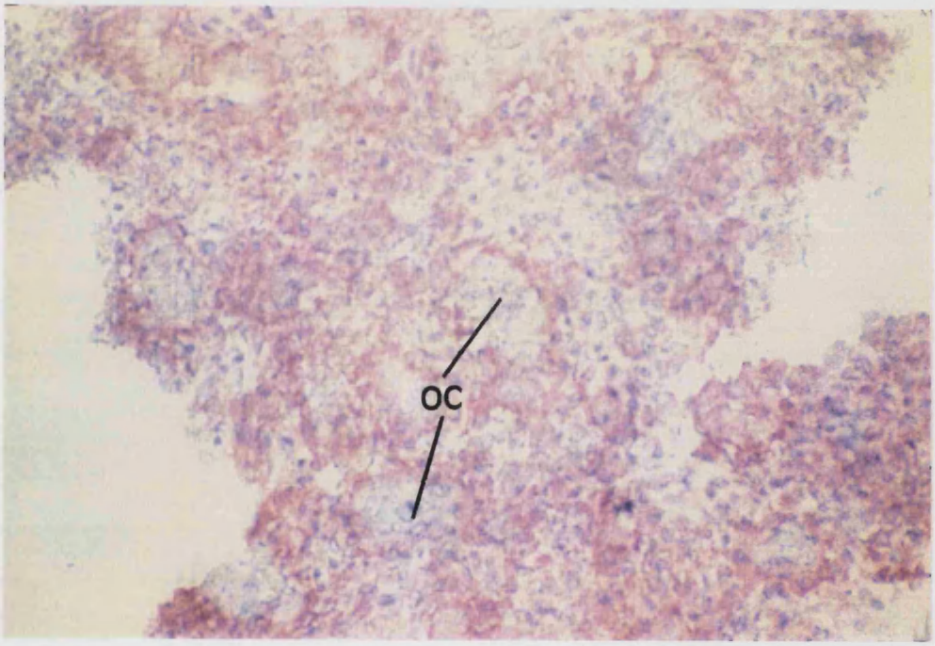
C



D



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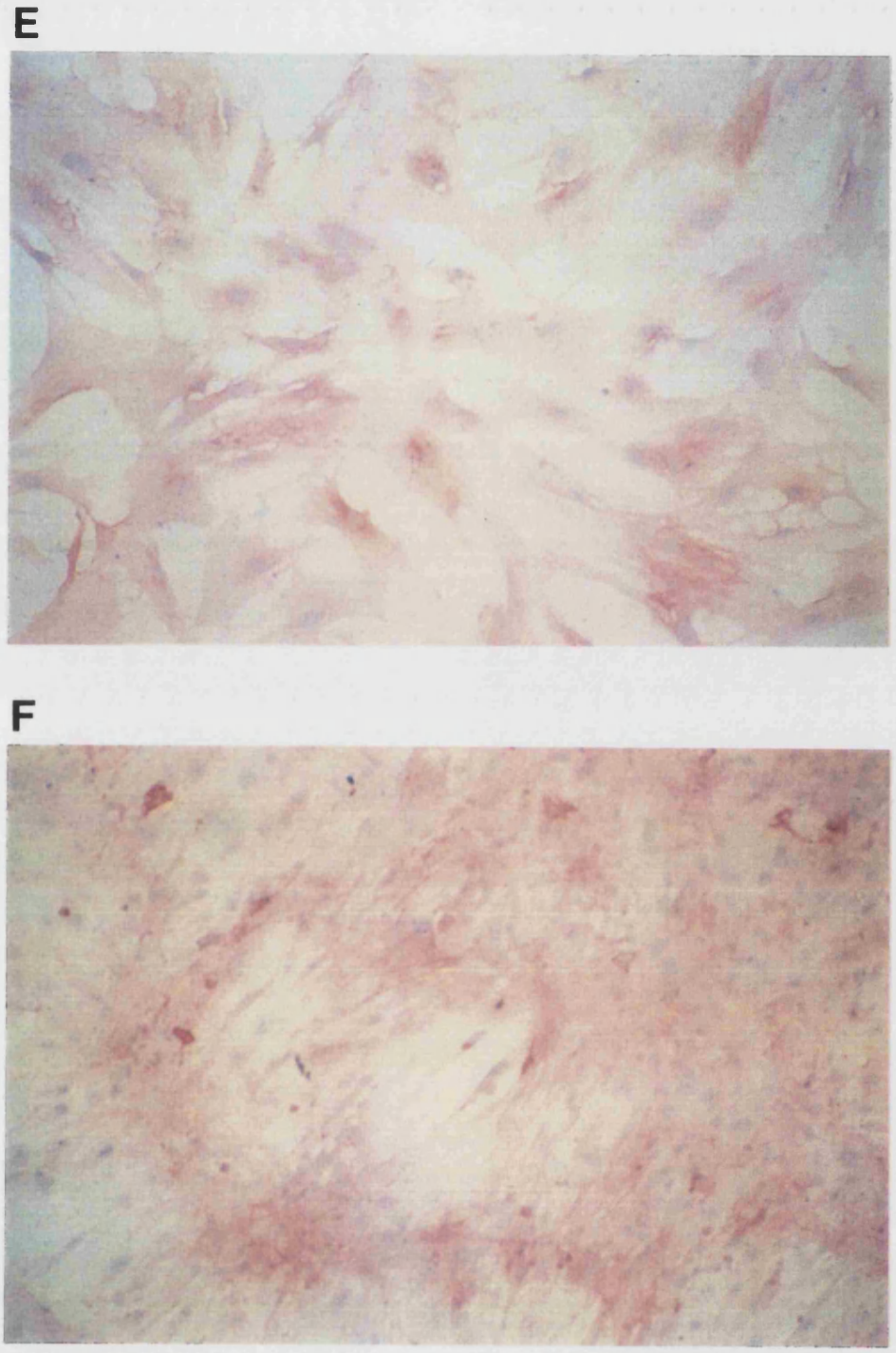


Figure 4.4 Expression of α3 subunits in cryostat sections of undecalcified human osteophyte, in sections of osteoclastoma and in cultured osteoblast-like cells.

Note positive staining of osteoblasts in areas of active/recent bone formation (A; OB) but differential staining of osteoblast lining cells (B; OBL). No expression is observed on osteoclasts (C; OC) which stain strongly for aV (D; positive control). Expression is maintained in culture (E). Staining patterns observed for α1 subunits are very similar but expression in cultured osteoblast-like cells is only readily apparent in confluent cultures (F).
Original magnification: A, B, C, D, E, F x 200. Bar, 45 μm

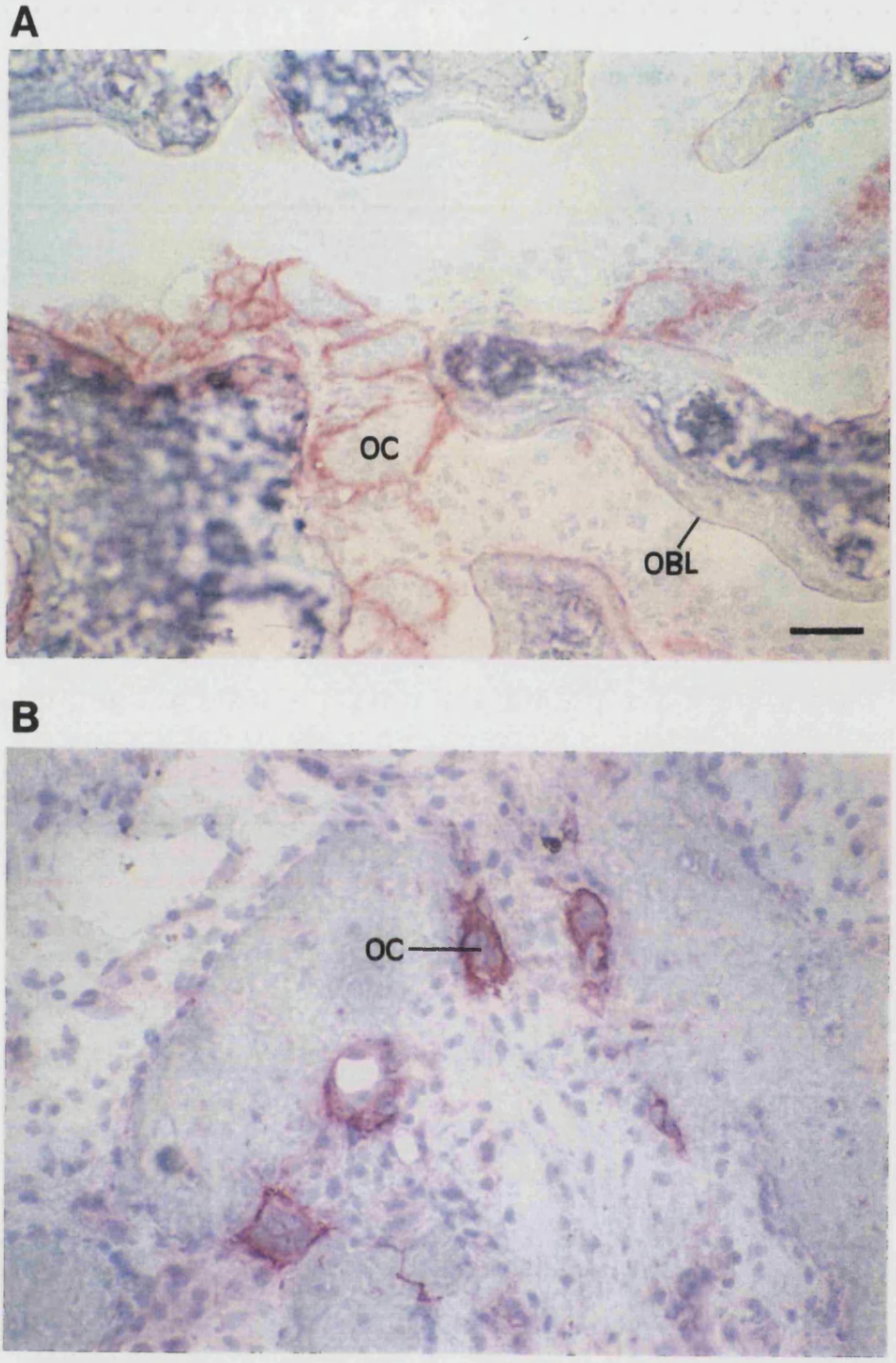
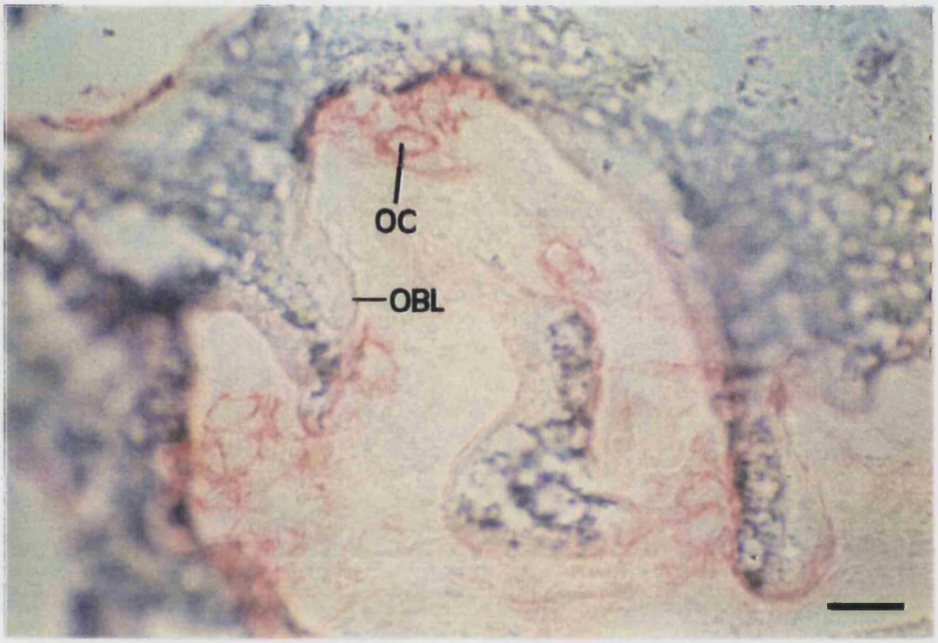


Figure 4.5 Expression of αV (A) and β3 (B) in cryostat sections of undecalcified human osteophyte.

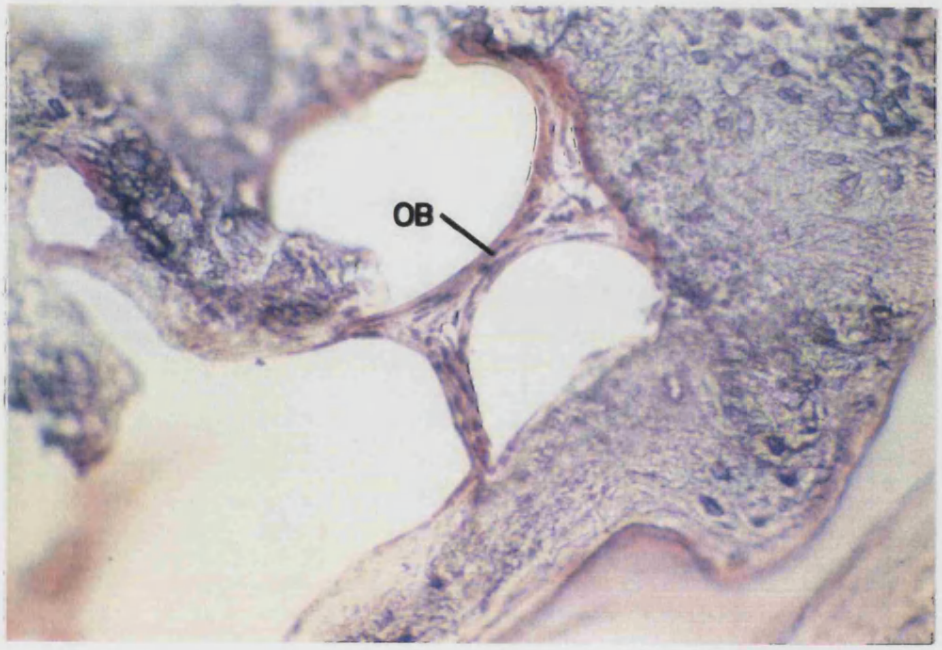
Osteoclasts are positive (OC) but osteoblast lining cells (OBL) which cover the bone surface are negative. Original magnification: A, B x 200. Bar, 45 μm

Figure 4.6

A



B



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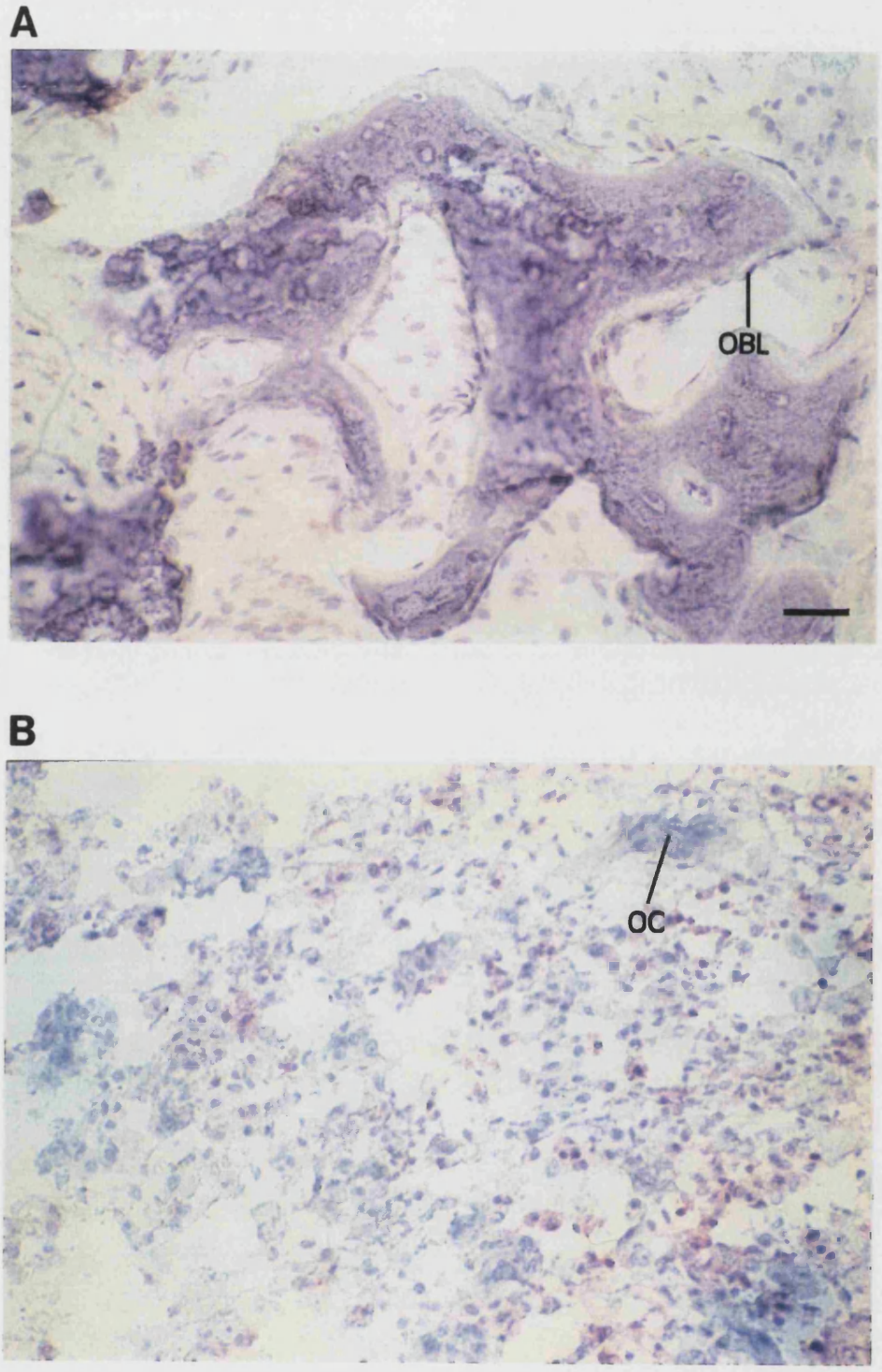
C

Figure 4.6 Expression of α2 in cryostat sections of undecalcified human osteophyte and cultured osteoblast-like cells.

Osteoclasts are positive (A; OC) but osteoblast lining cells are negative (A; OBL). Some osteoblastic staining is observed in areas of active/recent bone formation (B; OB) and expression is also observed in culture (C).
Original magnification: A x 100; B x 200; C x 100. Bar, 45 μm

Figure 4.7



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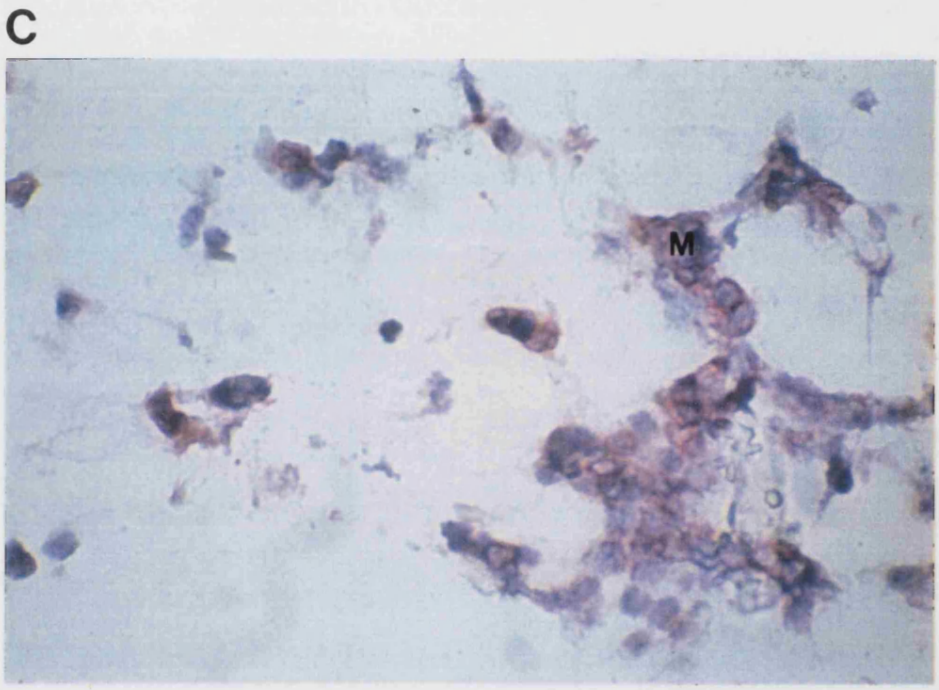


Figure 4.7 Expression of β2 in cryostat sections of undecalcified human osteophyte and sections of osteoclastoma.

Osteoblast lining cells which cover the bone surface are negative (A; OBL) and osteoclasts (B; OC) are negative, but there is positive staining in the marrow (C; M). Original magnification: A, B x 200; C x 400. Bar, 45 μm

CHAPTER 5.

REGULATION OF INTEGRIN SUBUNIT EXPRESSION ON HUMAN OSTEOBLAST-LIKE CELLS

5.1 Abstract

The aim of this study was to examine the expression of integrin subunits on human osteoblast-like cells by flow cytometry, and to determine whether expression of the major subunits was modulated by agents known to affect other aspects of human bone cell function. For these studies, experiments were performed to assess the effects of 1,25D, IL-1β and TGFβ, and growth on fibronectin, laminin, collagen I film and collagen I gels, on integrin subunit expression. Any modulation of integrin subunit expression was compared with other phenotypic effects elicited by these agents, and the significance of any correlations was assessed.

Under basal conditions, the major integrin subunits expressed by human osteoblast-like cells were α3 and β1 although α1 and α2 subunits were also present at relatively high concentrations. Treatment of human osteoblast-like cells with 1,25D (10^{-8}M) for 24-72 hr did not affect the expression of α1, α3, or β1 subunits, but expression of α2 was statistically increased after 72 hr. Administration of IL-1β (10 U/ml) for similar time periods, exerted pronounced effects on integrin subunit expression. Expression of α1 and α2 subunits was increased at all time points and levels of β1 were elevated after 48 and 72 hr. Expression of α3 subunits was increased after 24 hr but decreased after 48 and 72 hr. Another cytokine, TGFβ (25 ng/ml), also influenced integrin subunit expression. Like IL-1β, this cytokine increased expression of α2 at all time points and levels of β1 were elevated after 48 and 72 hr. Unlike the actions of IL-1β however, expression of α3 subunits in TGFβ treated cells was not significantly different from controls after 48 and 72 hr but decreased after 24 hr. In addition, α1 subunit expression was not affected following TGFβ administration.

Culture of human osteoblast-like cells on ECM components for a period of 10 days also influenced integrin subunit expression. Growth on fibronectin (5 μg/cm^2) and laminin (10 μg/cm^2) did not dramatically affect expression of α1, α2, α3 or β1 subunits. However, expression of α3 was statistically increased by fibronectin, and conversely, expression of α1, α3 and β1 were significantly reduced by laminin. Culture on collagen I gels (2.5 mm depth) exerted profound effects on integrin subunit expression, the most pronounced of which was a 3.5 fold increase in the expression of α2. Other changes included an increase in expression of α1 and β1 and a decrease in expression of α3. The effects of collagen I film (10 μg/cm^2) were different to those of collagen I gels: growth on this matrix decreased expression of all the major integrin subunits but particularly α1 which was reduced by over 50%.

The modulation of integrin subunit expression exhibited by cytokines and different matrix components could not be correlated with changes in proliferation, alkaline phosphatase activity or osteocalcin production. However, expression of α2 subunits was directly associated with gel contraction and corresponding changes in cell morphology, which implies that different integrin subunits exert specific effects.

5.2 Introduction

Apart from mediating cell adhesion, the integrin molecules play an important role in transmitting signals between the external environment and the interior of the cell. One such mechanism by which this communication could occur is through changes in the level of integrin subunits expressed. Many soluble mediators have been shown to affect integrin subunit expression in non bone cell types and interestingly, some of these, namely IL-1β, TGFβ, 1,25D, glucocorticoids, retinoic acid, TNFα and IFNγ, also influence the actions of osteoblasts and osteoclasts (see Introduction). In view of their direct effects on the phenotypic characteristics of different cell types (Chapter 3), ECM components themselves are also likely to modulate integrin receptor function.

Very little information is available concerning the regulation of integrin subunit expression in normal adult human bone. A few experiments have been performed *in vitro* using osteosarcoma cell lines but at present there is no published data from bone cell cultures. The aim of this study was to examine the expression of integrin subunits on human osteoblast-like cells by flow cytometry (FACS analysis), and determine whether expression of the major integrin subunits was modulated by agents known to affect other aspects of bone cell function. For these experiments cells were cultured over a period of 1-3 days, in the presence and absence of three soluble mediators: 1,25D, IL-1β and TGFβ; each at a concentration known to produce a functional response. In light of the results obtained following growth on the different matrix components (Chapter 3), cells were also cultured on collagen I, fibronectin and laminin for a period of 10 days and integrin subunit expression was compared to levels obtained following growth on plastic. A modulation of integrin subunit expression was compared with other phenotypic effects elicited by these agents, and the significance of any correlations was assessed.

These studies required very large numbers of bone cells and in view of their slow growth rate (4-5 days) and the difficulty of obtaining sufficient bone for the establishment of cultures, all experiments were performed using just one reagent concentration over a very specific time period. 1,25D is a standard differentiating agent which decreases cell proliferation and induces alkaline phosphatase activity and osteocalcin production in human bone-derived cells over a period of 24-72 hr (Beresford et al.1984b; Skjodt et al.1985; Beresford et al.1986); it was used at the optimum concentration of 10^{-8}M in the presence of 5% CS-FCS. Il-1β acts on human osteoblast-like cells over a similar time period, and increases proliferation (Gowen et

al.1985; Gowen, 1988; Evans et al.1989), plasminogen activator activity (Evans et al.1989) and prostaglandin synthesis (Gowen, 1988; Evans et al.1989). In addition, it reduces basal and 1,25D stimulated alkaline phosphatase activity (Evans et al.1989) and decreases 1,25D induced osteocalcin production (Beresford et al.1984b). This agent was used at the optimum concentration of 10 U/ml (10^{-12}M) in the presence of 3% CS-FCS. The effects of TGFβ on osteoblast-like cells are complex and dependent on the experimental model chosen for investigation. Centrella et al.(1987) showed that the TGFβ (0.15-15 ng/ml) increased proliferation when added to confluent cultures of rat osteoblast-like cells in serum free medium for 23 hr. However, these effects were biphasic and dependent on cell density and concentration of TGFβ applied. In contrast, Pfeilschifter et al.(1987) showed that similar concentrations of TGFβ (0.1-10 ng/ml) inhibited proliferation when added to rat osteosarcoma cells (ROS 17/2.8) for 48 hr in the presence of 5% FCS or 1 mg/ml BSA. These authors also demonstrated enhanced alkaline phosphatase activity following treatment with TGFβ and in both experimental models, collagen synthesis was increased. In order to assess the effects of TGFβ on human bone-derived cells, it was decided to use a concentration of 25 ng/ml TGFβ in the presence of 3% CS-FCS. The concentration of TGFβ applied was higher than that used in other studies, but the actual concentration added is likely to be less than 25 ng/ml: TGFβ is a labile, sticky peptide and therefore small amounts will be lost through denaturation and attachment to the storage vessel. It would have been better to perform all these studies in serum free medium to prevent test agents acting in concert with other growth factors present within serum. However, cells were very unhealthy after 72 hr under these conditions and therefore all experiments were performed in the presence of small amounts of CS-FCS.

In order to assess the effects of ECM components on integrin subunit expression, human osteoblast-like cells were cultured on collagen I, fibronectin and laminin under conditions known to exert maximal effects on cell phenotype (Chapter 3). Cells were therefore cultured on 10 μg/cm^2 laminin, 10 μg/cm^2 collagen I film and 2.5 mm gels for a period of 10 days. Growth on fibronectin exerted little effect on cell phenotype but cells were cultured on 5.0 μg/cm^2 fibronectin to determine whether this matrix affected the levels of integrin subunits expressed. Any modulation of integrin subunit expression following growth on the different ECM components was correlated with the phenotypic effects reported in Chapter 3.

5.3 Methods

5.3.1 Effect of collagenase I on integrin subunit expression

Human osteoblast-like cells were cultured for 72 hr and then half the cells were detached by trypsinisation (Method 2.2.5) and prepared for FACS analysis (Method 2.6). The remaining cells were pre-incubated with collagenase I (0.3% in serum free medium) for 30 min, then detached with trypsin and prepared for FACS analysis. MFI values obtained for individual integrin subunits were converted to receptor sites/cell and the effects of collagenase were determined.

5.4 Results

5.4.1 Detection of integrin subunits by FACS analysis

Accurate assessment of integrin subunit expression by flow cytometry requires single cell suspensions. Initially this was achieved by scraping bone-derived cells off the bottom of the tissue culture dish but this technique was very damaging and produced clumped cell preparations which were difficult to analyse. An alternative approach was to detach cells enzymically using trypsin and therefore experiments were undertaken to compare the intensity of APAAP staining between cytospin preparations of trypsinised cells and cells cultured on glass slides. Both of these cell preparations produced similar staining patterns but the APAAP technique is not strictly quantitative and it was difficult to compare the intensity of staining produced due to differences in morphology: cells seeded onto multi-spot slides were spread out whereas those spun onto glass slides were rounded (data not shown). In light of the similar staining patterns observed, it was concluded that enzymic digestion with trypsin did not grossly affect antigenic detection. Therefore this method was used in all subsequent experiments for the preparation of single cell suspensions for FACS analysis.

In order to assess the integrin subunit profile of bone-derived cells, cultures of human osteoblast-like cells were prepared for FACS analysis (Method 2.6) using all the anti-integrin mAbs to ensure that subunits detected by FACS analysis were similar to those identified in previous immunolocalisation studies. When analysing FACS results, a dot plot of side scatter (SSC) against forward scatter (FSC) was produced from negative control data and a region enclosing the majority of the population was selected for subsequent analysis (Fig. 5.1). Histograms for individual integrin subunits, of cell number against fluorescence, were then overlayed with data from appropriately diluted

negative controls, and the integrin subunit profile of human osteoblast-like cells was determined (Fig. 5.2).

The illustrations in Figure 5.2 clearly demonstrate that α1, α2, α3 and β1 were the major integrin subunits expressed by cultured human osteoblast-like cells: there was no staining with mAb raised against α4, α6, β2 and β3, and αV subunits were weakly expressed. All these observations correlate with results obtained in previous immunolocalisation studies (Chapter 4). The only discrepancy between results obtained by FACS and APAAP concerned expression of α5. This subunit was detected on bone derived cells by FACS at a 1/25 dilution of mAb but no staining was observed using APAAP. This may have resulted from the time of fixation and/or the type of fixative employed. When integrin subunits were identified by APAAP, cells and sections were fixed with acetone at the beginning of the procedure. However, when detected by FACS, cells were fixed with 1% para-formaldehyde at the end of the procedure. It is possible that fixation conditions required for the APAAP technique specifically mask antigenic expression of α5 in human osteoblast-like cells and therefore care should be taken when interpreting negative data obtained from immunolocalisation studies.

5.4.2 Titration of mAbs for antigenic detection

In order to accurately quantify the number of integrin receptor sites/cell, mAbs must be present at saturating concentrations. Therefore, cultures of osteoblast-like cells were prepared for FACS analysis (Method 2.6) using all the positive anti-integrin mAbs over a range of mAb concentrations. No such titration experiments were performed for mAbs directed against α5 and αV subunits: previous results demonstrated that anti-integrin α5 should be diluted 1/25, and anti-integrin αV should be added directly to cells without prior dilution. When analysing FACS data, MFI values were obtained for each sample and titration curves of fluorescence against mAb dilution were prepared (Fig. 5.3).

Based on results from titration experiments and relative availability, mAb dilutions used in subsequent FACS experiments are shown in Table 5.1.

Table 5.1 mAb dilutions used for FACS experiments

Integrin subunit detected	mAb	Optimum dilution of mAb
α1	T52/7	1/100
α2	G19	1/100
α3	PB15	1/50
a5	SAM1	1/25
aV	23C6	neat
β1	4B4	1/50

Human osteoblast-like cells were cultured for 72 hr and prepared for FACS analysis using anti-integrin mAbs (or equivalent concentrations of IgG) (Method 2.6). mAbs derived from ascites were diluted between 1/25 and 1/3200 but mAbs from tissue culture supernatants were not diluted prior to use. MFI values were used to plot a titration curve and optimum concentrations of anti-integrin mAbs were determined.

5.4.3 Quantitation of integrin subunit expression on human osteoblast-like cells

Human osteoblast-like cells were cultured for 72 hr and then prepared for FACS analysis using saturating concentrations of mAb or IgG (Method 2.6). MFI values obtained for each sample were converted to receptor sites/cell using results obtained from 6 standardised preparations of FITC-coated beads. Under basal conditions, α3 and β1 were the major integrin subunits expressed although α1 and α2 subunits were also present at relatively high concentrations (Table 5.2). In subsequent experiments it was therefore decided to study the expression of these four integrin subunits: αV subunit expression was not further pursued due to the low values obtained for receptor sites/cell. Expression of α5 was not further investigated due to the high concentration of mAb required for its detection and the negative staining patterns obtained in previous immunolocalisation studies (Chapter 4).

Table 5.2. A quantitative comparison of the integrin subunits expressed by human osteoblast-like cells

Integrin subunit detected	Mean receptor sites/cell (standard error)
α1	101023 (6077)
α2	101866 (6707)
α3	127184 (5134)
α4	negative
α5	47544 (3735)
α6	negative
αV	15561 (1701)
β1	556947 (26167)
β2	negative
β3	negative

Human osteoblast-like cells were cultured for 72 hr and prepared for FACS analysis using saturating concentrations of mAb (or IgG) (Method 2.6). MFI values were converted to receptor sites/cell and a minimum of 9 different values were pooled to quantify integrin subunit expression.

5.4.4. Modulation of integrin subunit expression by 1,25D, IL-1β and TGFβ

Confluent cultures of primary human osteoblast-like cells were treated with test agents for 24-72 hr (Method 2.4.6) and prepared for FACS analysis using saturating concentrations of mAb (or equivalent concentrations of IgG) (Method 2.6). At each time point, cells were also assayed (in triplicate) for effects of the test agents on alkaline phosphatase activity (Method 2.4.2), osteocalcin production (Method 2.4.4) (1,25D

treated groups only) and in the IL-1β and TGFβ treated groups, cell proliferation (Method 2.4.1). The modulation of integrin subunit expression following treatment with 1,25D, IL-1β and TGFβ is illustrated in Figs. 5.4, 5.6 and 5.8, and the typical effects of these agents on other aspects of bone cell function, are summarised in Figs. 5.5, 5.7 and 5.9.

Values obtained for integrin subunit sites/cell in control groups remained constant throughout the treatment period. Addition of 1,25D consistently increased alkaline phosphatase activity and osteocalcin production (Fig. 5.5) but did not affect the expression of α1, α3 or β1 subunits (Fig. 5.4). After 72 hr however, there was a small but statistically significant increase in expression of α2 (Fig. 5.4B). In contrast to 1,25D, IL-1β exerted dramatic effects on the number of integrin subunits expressed (Fig. 5.6). The most noticable of these concerned expression of α1 and α2 subunits which were significantly increased at all time points (Fig. 5.6A and B). The expression of β1 subunits was also increased, but not to the same extent as those for α1 and α2 and only after 48 and 72 hr (Fig. 5.6D). α3 subunit expression was increased by treatment with IL-1β at 24 hr, but decreased at 48 and 72 hr (Fig. 5.6C). Addition of TGFβ also modulated integrin subunit expression (Fig. 5.8). The most pronounced effect concerned expression of α2 subunits, which were significantly enhanced at 24 hr and further increased after 48 and 72 hr (Fig. 5.8B). Expression of β1 subunits was also increased after 48 and 72 hr but the effect was less marked than that observed for α2 (Fig. 5.8D). Expression of α3 subunits was not significantly different from controls at 48 and 72 hr but statistically decreased after 24 hr (Fig. 5.8C). Expression of α1 subunits was not significantly altered following administration of this cytokine (Fig. 5.8A).

The modulation of integrin subunit expression in response to IL-1β and TGFβ could not be correlated with effects on cell proliferation or alkaline phosphatase activity (Fig. 5.7 and Fig. 5.9): neither of these agents significantly influenced cell number over 24-72 hr and both exerted different effects on cellular alkaline phosphatase.

5.4.5 Modulation of integrin subunit expression by different ECM components

Human osteoblast-like cells were cultured on plastic, fibronectin (5 μg/cm^2), laminin (10 μg/cm^2), collagen I film (10 μg/cm^2) and collagen I gels (2.5 mm depth; 10 ml) (Method 2.4.5). After 10 days, cells were detached by enzymic digestion with trypsin or collagenase (Method 2.2.5), prepared for flow cytometry and analysed as described

(Method 2.6). Preliminary experiments showed that treatment of human osteoblast-like cells with collagenase did not affect integrin subunit detection (Table 5.3). The effects of culturing human osteoblast-like cells on different matrix components are illustrated in Fig. 5.10. Growth on 5 $\mu g/cm^2$ fibronectin and 10 $\mu g/cm^2$ laminin did not dramatically alter expression of $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\beta 1$ subunits (Fig. 5.10A and B). However, fibronectin statistically increased expression of $\alpha 3$ subunits and laminin significantly decreased expression of $\alpha 1$, $\alpha 3$ and $\beta 1$. Culture of human osteoblast-like cells on collagen I film exerted more pronounced effects on integrin subunit expression (Fig. 5.10C): $\alpha 1$ subunits were decreased by more than 50% and expression of $\alpha 2$, $\alpha 3$ and $\beta 1$ were also significantly reduced. Growth on collagen I gels typically altered cell morphology, promoted gel contraction (as described in Chapter 3) and profoundly influenced integrin subunit expression (Fig. 5.10D). The most striking effect was observed with $\alpha 2$, expression being increased by 3.5 fold: other effects included an increased expression of $\alpha 1$ and $\beta 1$ and a decreased expression of $\alpha 3$. The fact that collagen I film and collagen I gels exhibited different effects suggests that the organisation of collagen fibres within the ECM is important for the regulation of integrin subunit expression.

Table 5.3. Effect of collagenase digestion on integrin subunit detection

	Mean number receptor sites/cell (standard deviation) (n=3)	
Integrin subunit detected	Control	30 min collagenase digestion
$\alpha 1$	107094 (3720)	105650 (2804)
$\alpha 2$	101005 (3245)	102192 (2629)
$\alpha 3$	130383 (9787)	139664 (2021)
$\beta 1$	547060 (16875)	528445 (17104)

Human osteoblast-like cells were cultured for 72 hr and half the cells were pre-incubated with collagenase I (30 min/ 37°C) before detachment by trypsinisation. Cell suspensions were then prepared for FACS analysis using saturating concentrations of mAb (or IgG). MFI values were converted to receptor sites/cell and the effects of collagenase on integrin subunit detection were assessed.

5.5 Discussion

Under basal conditions, α3 and β1 were the primary integrin subunits expressed which can combine to form α3β1, a receptor which is commonly found in cells from most tissues and binds collagen , fibronectin and laminin (Humphries, 1990). α1 and α2 subunits were also expressed at relatively high concentrations. These subunits associate with β1 to form receptors for collagen and laminin (Humphries, 1990).

The effects of IL-1β on integrin subunit expression generally correlated with previous findings obtained using MG-63 osteosarcoma cell lines. For example, Dedhar (1989) showed that IL-1β (5 pM; 20 hr) increased expression of the integrin α5β1 by 6-10 fold and Milam et al.(1991) demonstrated that treatment with IL-1β (10 pM, 48 hr) modulated the expression of multiple integrin subunits: the greatest response occurred with α2 subunit expression which was elevated 3.3 fold. In addition, Santala and Heino, (1991) showed that IL-1β (10 U/ml, 22 hr), increased expression of α1 and decreased expression of α3; however, in this report IL-1β exerted no effect on expression of β1. The effects of TGFβ on integrin subunit expression of human osteoblast-like cells also correlated with studies performed using MG-63 cells. For instance, Heino and Massague (1989) showed that addition of TGFβ (5 ng/ml; 12-24 hr in serum free medium) elevated levels of α2 and β1 and decreased expression of α3 subunit mRNA. Results obtained from osteosarcoma cell lines were often observed over a shorter time period and effects were more dramatic than those displayed by normal osteoblast-like cells. These differences could result from the altered growth properties exhibited by transformed cells. Alternatively, the low serum concentrations present in experiments utilising bone-derived cells could affect their responsiveness to TGFβ.

Studies documenting the effects of 1,25D on cell-matrix interactions in bone cell types have mainly been concerned with effects on ECM synthesis. In these studies, expression of α2 was statistically increased but only after 72 hr, a finding which could imply that the actions of 1,25D occur indirectly via induction of a bone cell product. In support of this, studies performed by other members of our laboratory have demonstrated that addition of 1,25D increased TGFβ mRNA (Merry and Gowen, 1992): in light of the effects of TGFβ on integrin subunit expression it is possible that production of TGFβ in response to 1,25D is promoting increased expression of α2.

Although there have been no published data on the effects of 1,25D on integrin subunit expression of osteoblast-like cells, one report has demonstrated a 50 fold increase in

expression of αVβ3 following addition of 1,25D to osteoclast precursor cells (Medhora et al.1993). 1,25D is known to increase synthesis of ECM proteins such as osteopontin in bone-derived cells (Prince and Butler, 1987). Osteopontin is a potential ligand for the integrin molecule αVβ3 and it is therefore possible that production of ECM proteins and modulation of integrin subunit expression act together to regulate the bone remodelling cycle.

Each of the FACS experiments was performed in conjunction with other assays in an attempt to correlate regulation of integrin subunit expression with modulation of osteoblast phenotype. 1,25D treated cells exhibited a time dependent increase in alkaline phosphatase activity and osteocalcin production, and in previous reports, this has been correlated with a decrease in cell proliferation (Beresford et al.1986). In these studies however, it was not possible to show that the decrease in proliferation and enhancement of differentiation were associated with changes in integrin subunit expression. Experiments monitoring changes in proliferation and alkaline phosphatase activity were performed using cells treated with IL-1β and TGFβ. Neither of these agents increased cell number over 72 hr. This finding may have resulted from the slow doubling time (4-5 days) of osteoblast-like cells and longer incubation periods would probably be required for a proliferative response. Alternatively, the proliferative effects of IL-1β could be inhibited by prostaglandin production (Gowen, 1988; Evans et al.1989) and indomethacin (1 μM) should perhaps have been incorporated into the experimental assay. In contrast to the similar effects on cell proliferation, IL-1β and TGFβ both elicited different effects on cellular alkaline phosphatase activity. From these studies, the functional significance of a modulation of integrin subunit expression is therefore difficult to ascertain.

It was shown earlier (Chapter 3) that growth on a variety of ECM components affected four different aspects of osteoblast function. Therefore cells were cultured under these conditions to determine whether there were any corresponding changes in integrin subunit expression. The minimal effects of fibronectin (5 $\mu g/cm^2$) on integrin subunit expression correlate with the minor effects of this substrate on proliferation and expression of a differentiated osteoblast phenotype (Chapter 3). As mentioned previously, this can partly be explained by the high concentrations of fibronectin present within serum which would make conditions in control and fibronectin coated plates very similar. In previous experiments, growth on laminin (10 $\mu g/cm^2$), collagen I film (10 $\mu g/cm^2$) and collagen I gels (2.5 mm depth) reduced alkaline phosphatase activity and osteocalcin production. The fact that these three matrices exerted different effects on integrin subunit expression suggests that integrin subunit expression and

induction of a differentiated osteoblast phenotype are not intimately associated events. A major difference observed between growth of human osteoblast-like cells on collagen I film and collagen I gels was that of morphology. Cells cultured on collagen I film looked very similar to those grown on plastic. However, cells cultured on collagen I gels produced long cellular extensions which penetrated the gel, and this was associated with gel contraction. Cells cultured on collagen I gels exhibited a 3.5 fold increase in expression of $\alpha 2$ which was not observed following growth on collagen I film. This suggests that the $\alpha 2$ subunit is concerned with mediating gel contraction and that the different cytoskeletal interactions which result, lead to the characteristic changes in cell morphology. This finding agrees well with studies performed by Schiro et al.(1991) who demonstrated that the integrin molecule $\alpha 2\beta 1$ mediated reorganisation and contraction of collagen matrices. The involvement of the $\alpha 2$ subunit was later confirmed by Chan et al.(1992) who used chimeric constructs to demonstrate that the cytoplasmic domain of the $\alpha 2$ subunit specifically promoted gel contraction. This data suggests that integrins not only transduce signals from the matrix to the cytoskeleton, but can also be used by the cell to modify the tertiary structure of the matrix. This could have important implications for the process of bone remodelling.

Figure 5.1A.

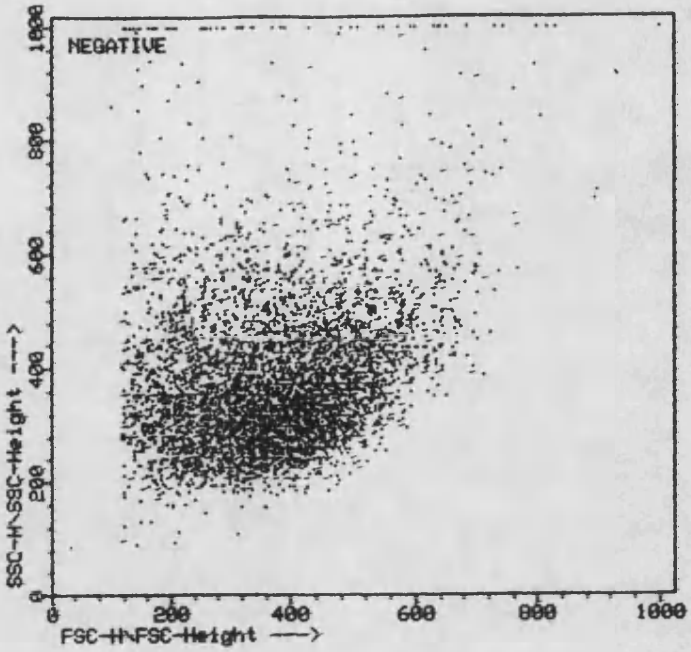


Figure 5.1B

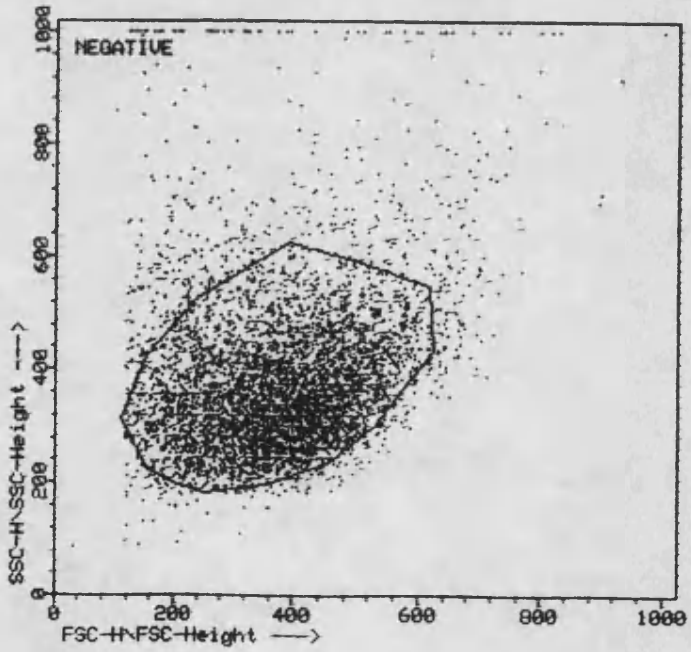


Figure 5.1 A typical population of human osteoblast-like cells prepared for FACS analysis.

Dotplots of side scatter (SSC) against forward scatter (FSC) were produced from negative controls to assess the homogeneity of the cell population (A). Heterogeneous cells were excluded by selecting a region enclosing the majority of the population (B), and this was used for subsequent analysis.

Figure 5.2A

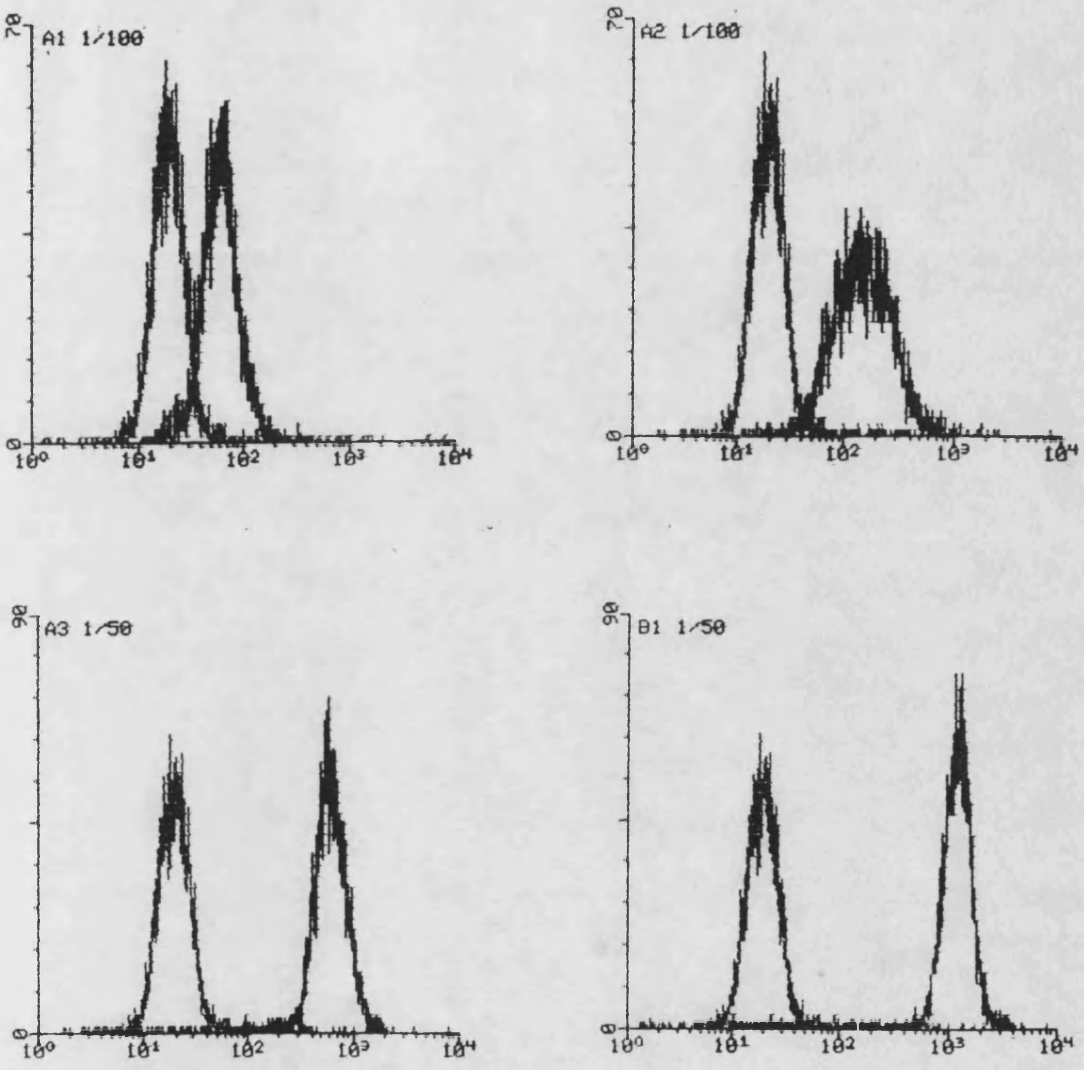


Figure 5.2 Detection of integrin subunit expression in human osteoblast-like cells by FACS analysis.

Human osteoblast-like cells were cultured for 72 hr and then prepared for FACS analysis using anti-integrin mAbs (or equivalent concentrations of IgG) (Method 2.6). mAbs derived from ascites were diluted 1/25, 1/50 and 1/100 but mAbs from tissue culture supernatants were not diluted prior to use. MFI values (abscissa) were plotted against cell no. (ordinate) and the integrin subunit profile was determined. The first peak represents negative control (IgG); the second peak represents the fluorescence obtained with anti-integrin mAbs.

Figure 5.2B

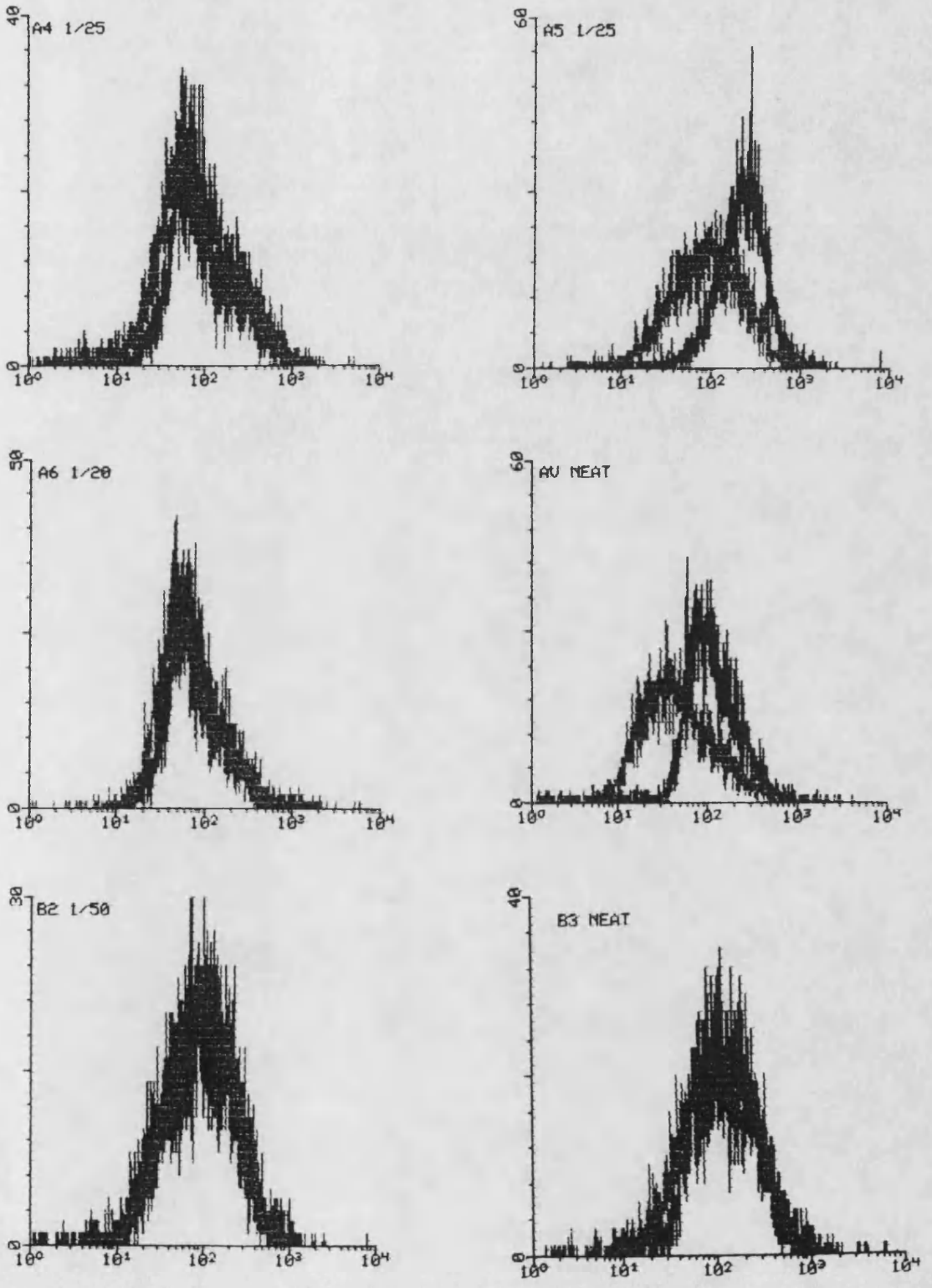
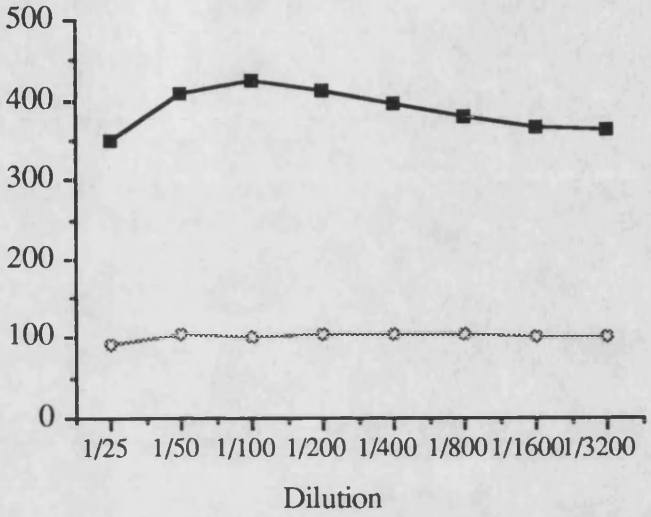


Figure 5.3

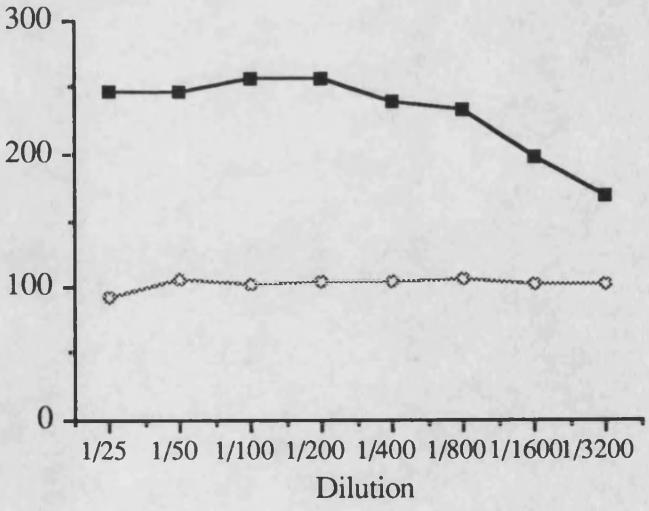
A. Titration α1 mAb



Mean fluorescence intensity

mAb
IgG

B. Titration α2 mAb



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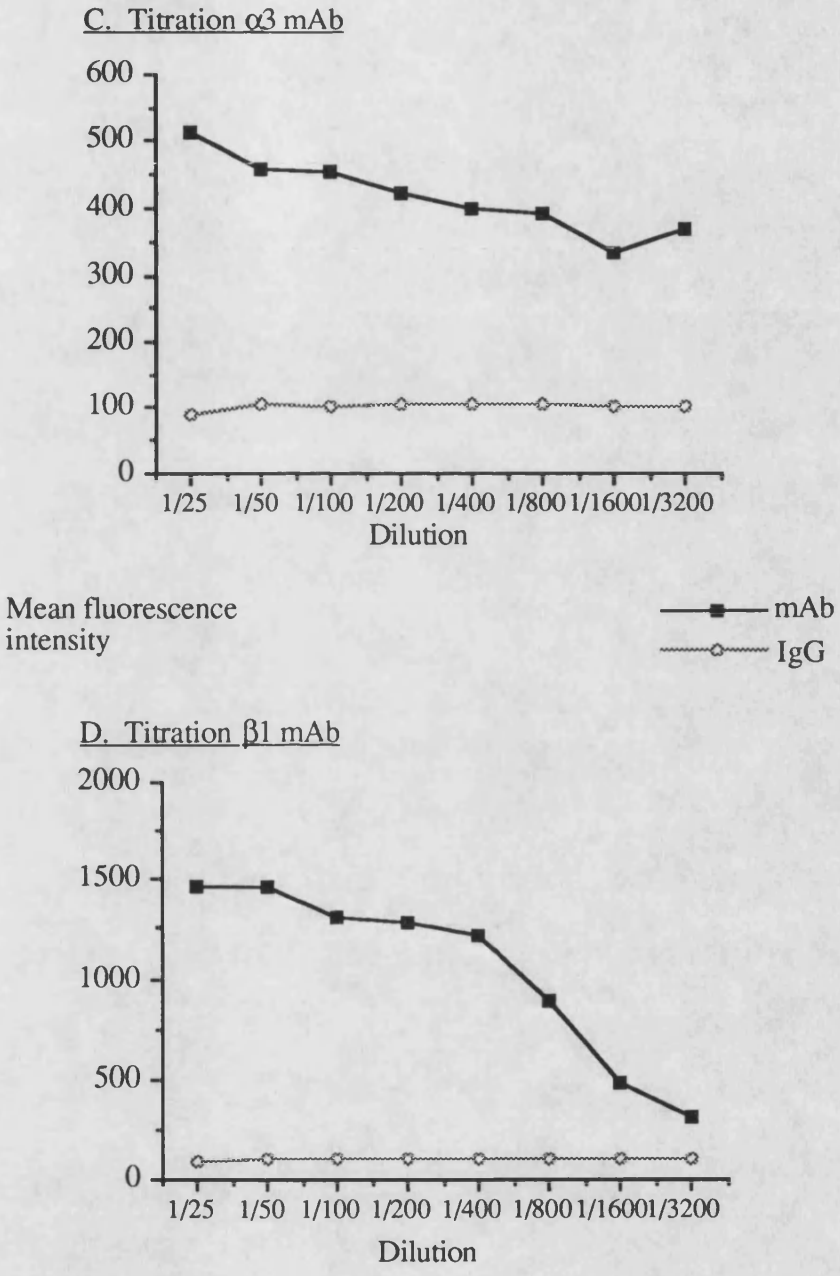
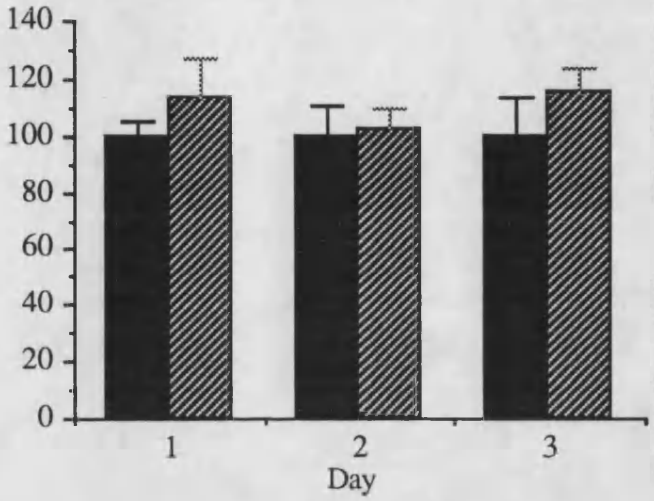


Figure 5.3 Titration of anti-integrin mAbs.

Human osteoblast-like cells were cultured for 72 hr and prepared for FACS analysis using anti-integrin α1 (A), α2 (B), α3 (C) and β1 (D) or equivalent concentrations of IgG. Values obtained for mean fluorescence intensity at each mAb dilution were then used to plot a titration curve and optimum concentrations of anti-integrin mAbs were determined.

Figure 5.4

A. α1 subunit expression (n=6)

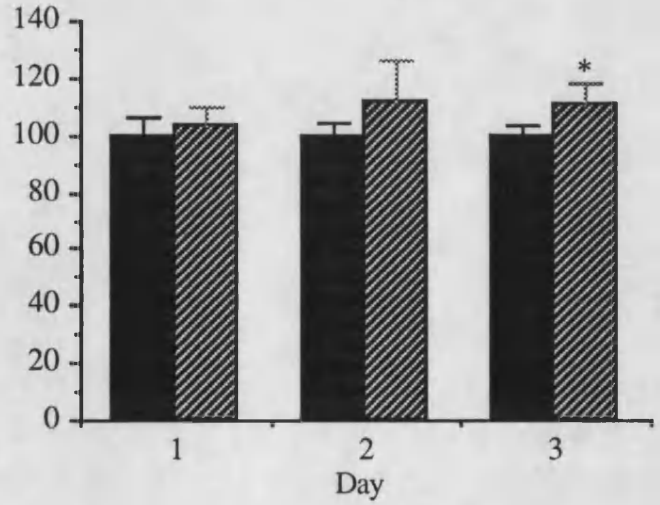


Integrin subunit expression as % control

Control
1,25D

* $p < 0.05$

B. α2 subunit expression (n=6)



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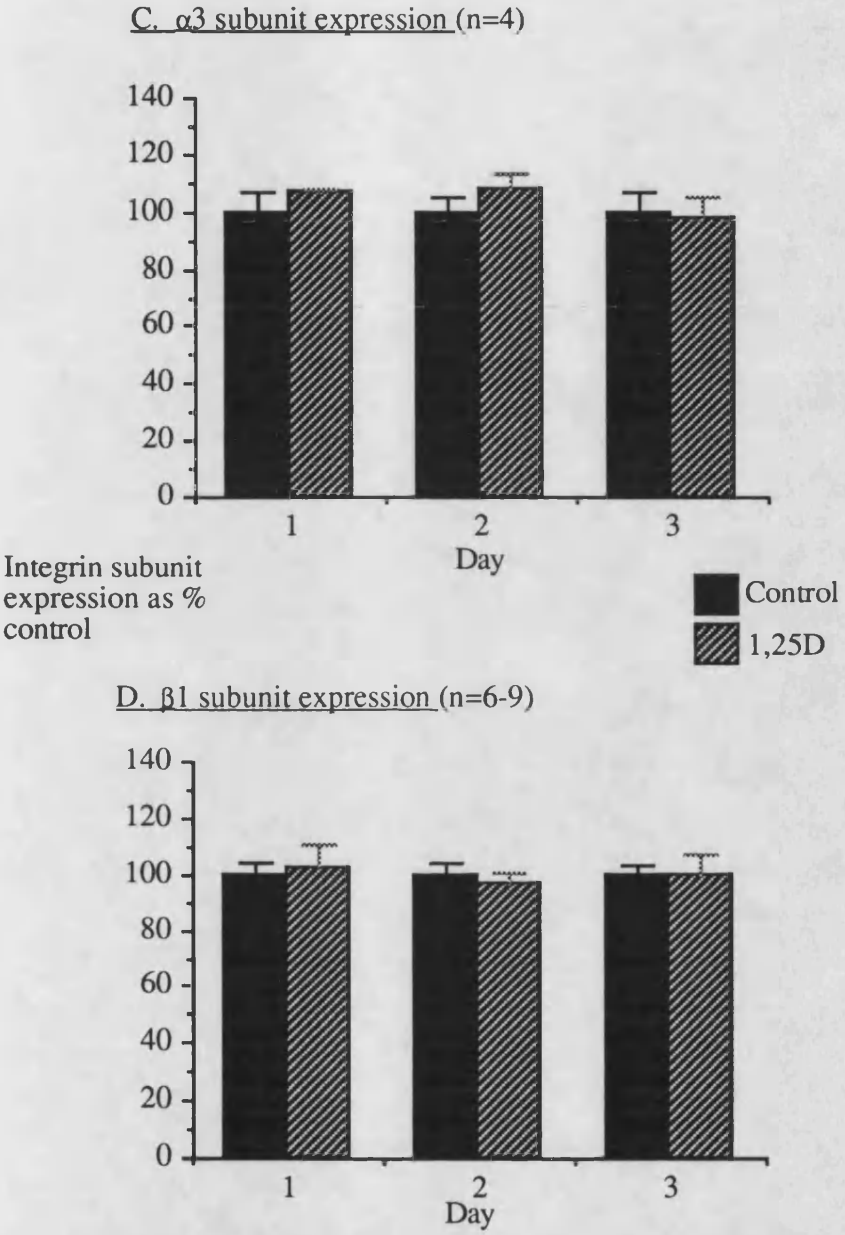


Figure 5.4 Modulation of integrin subunit expression by 1,25D.

Human osteoblast-like cells were treated with 1,25D (10^{-8}M) for 24, 48 and 72 hr and then prepared for FACS analysis using saturating concentrations of mAb (or IgG). MFI values were converted to receptor sites/cell and integrin subunit expression was represented as a % of control values i.e. the no. integrin subunits expressed by cells from control plates at each time period. Assays were undertaken in duplicate or triplicate and experiments were performed 2 or 3 times. All data were pooled and the significance of any modulation in expression of α1(A), α2 (B), α3 (C) and β1 (D) subunits was assessed using an unpaired Student's T-test.

Fig. 5.5A

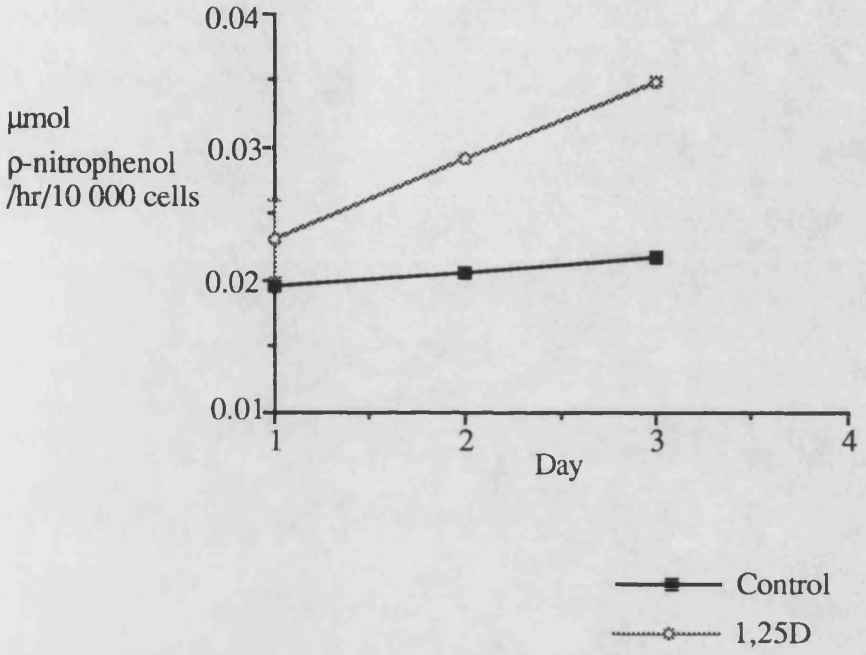


Fig. 5.5B

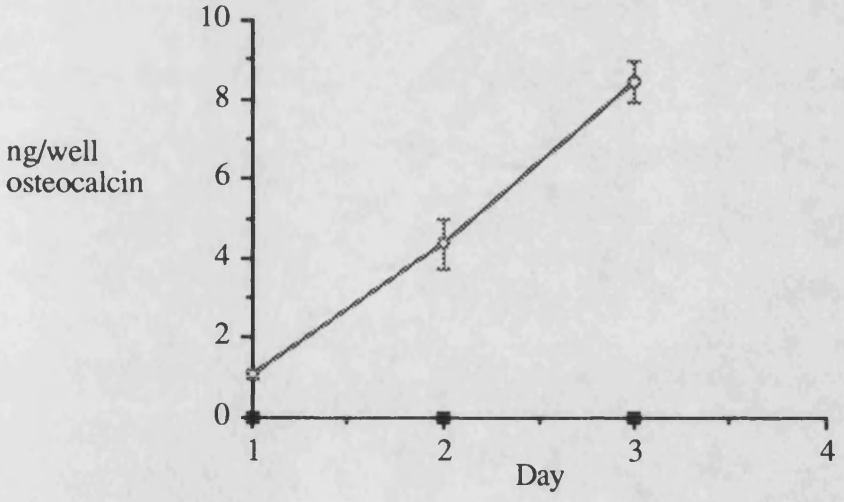
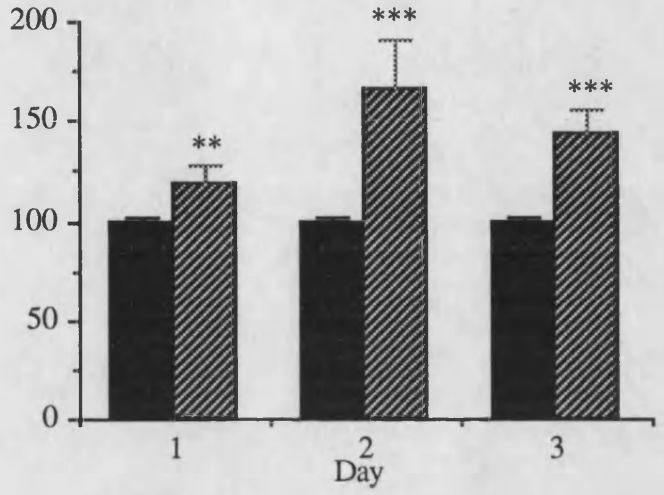


Figure 5.5 Effect of 1,25D on alkaline phospahatase activity and osteocalcin production.

Human osteoblast-like cells were treated with 1,25D (10^{-8}M) for 24-72 hr. At intervals of 24 hr, cells were assayed for alkaline phosphatase activity (A) and supernatants were assayed for osteocalcin production (B). Points are means and standard deviations from three samples and are representative of 3 experiments.

Figure 5.6

A. α1 subunit expression (n=6)

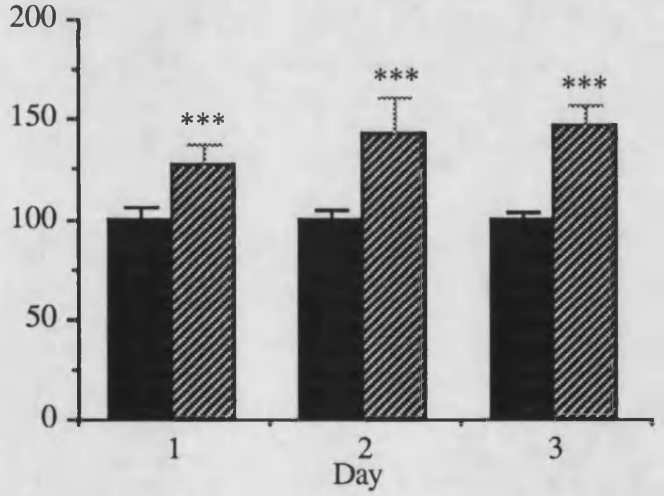


Integrin subunit expression as % control

Control
IL-1β

** $p < 0.01$
*** $p < 0.001$

B. α2 subunit expression (n=6)



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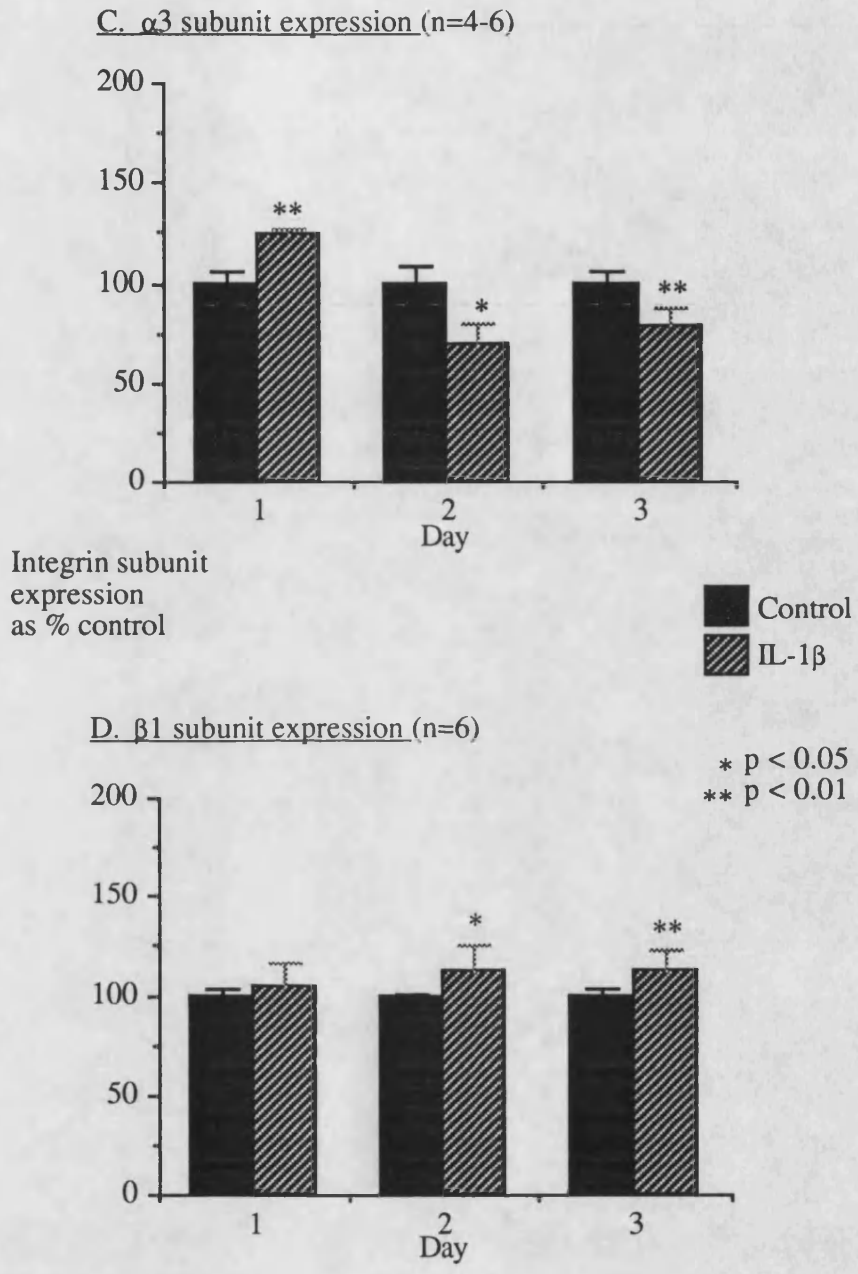


Figure 5.6. Modulation of integrin subunit expression by IL-1 β.

Human osteoblast-like cells were treated with IL-1 β (10 U/ml) for 24, 48 and 72 hr and then prepared for FACS analysis using saturating concentrations of mAb (or IgG). MFI values were converted to receptor sites/cell and integrin subunit expression was represented as a % of control values i.e. the no.integrin subunits expressed by control cells at each time period. Assays were undertaken in duplicate or triplicate and experiments were performed 2 or 3 times. All data were pooled and the significance of any modulation in expression of α1(A), α2(B), α3 (C) and β1(D) subunits was assessed using an unpaired Student's T-test.

Fig. 5.7A

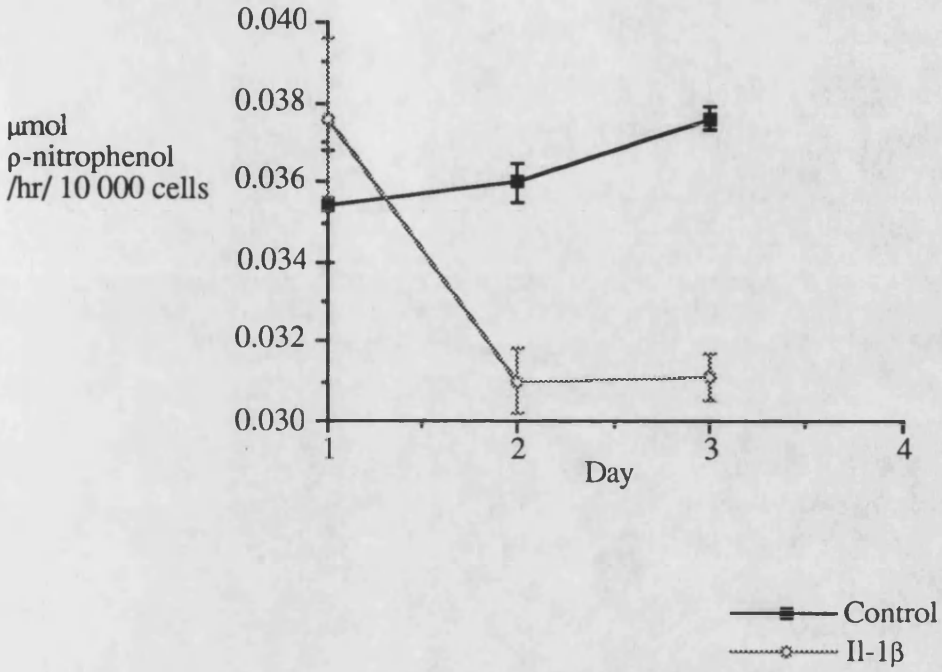


Fig. 5.7B

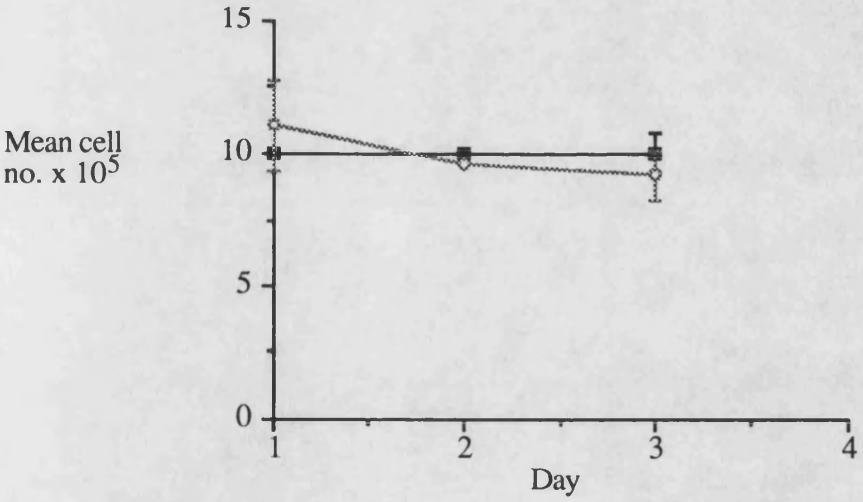
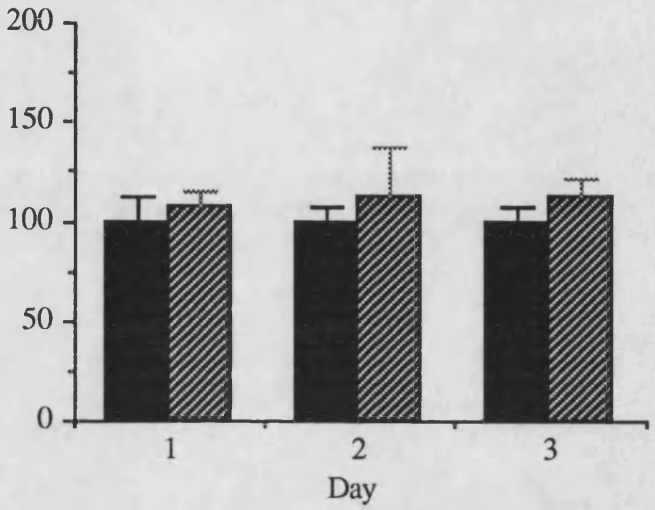


Figure 5.7 Effect of IL-1β on alkaline phospahatase activity and proliferation.

Human osteoblast-like cells were treated with IL-1 β (10 U/ml) for 24-72 hr. At intervals of 24 hr, cells were assayed for alkaline phosphatase activity (A) and proliferation (B). Points are means and standard deviations from three samples and are representative of 3 experiments.

Figure 5.8

A. α1 subunit expression (n=3-8)

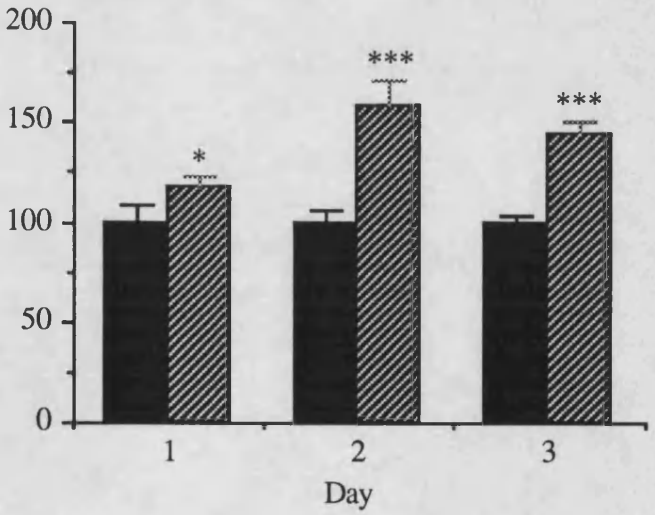


Integrin subunit expression as % control

Control
TGFβ

* $p < 0.05$
*** $p < 0.001$

B. α2 subunit expression (n=3-5)



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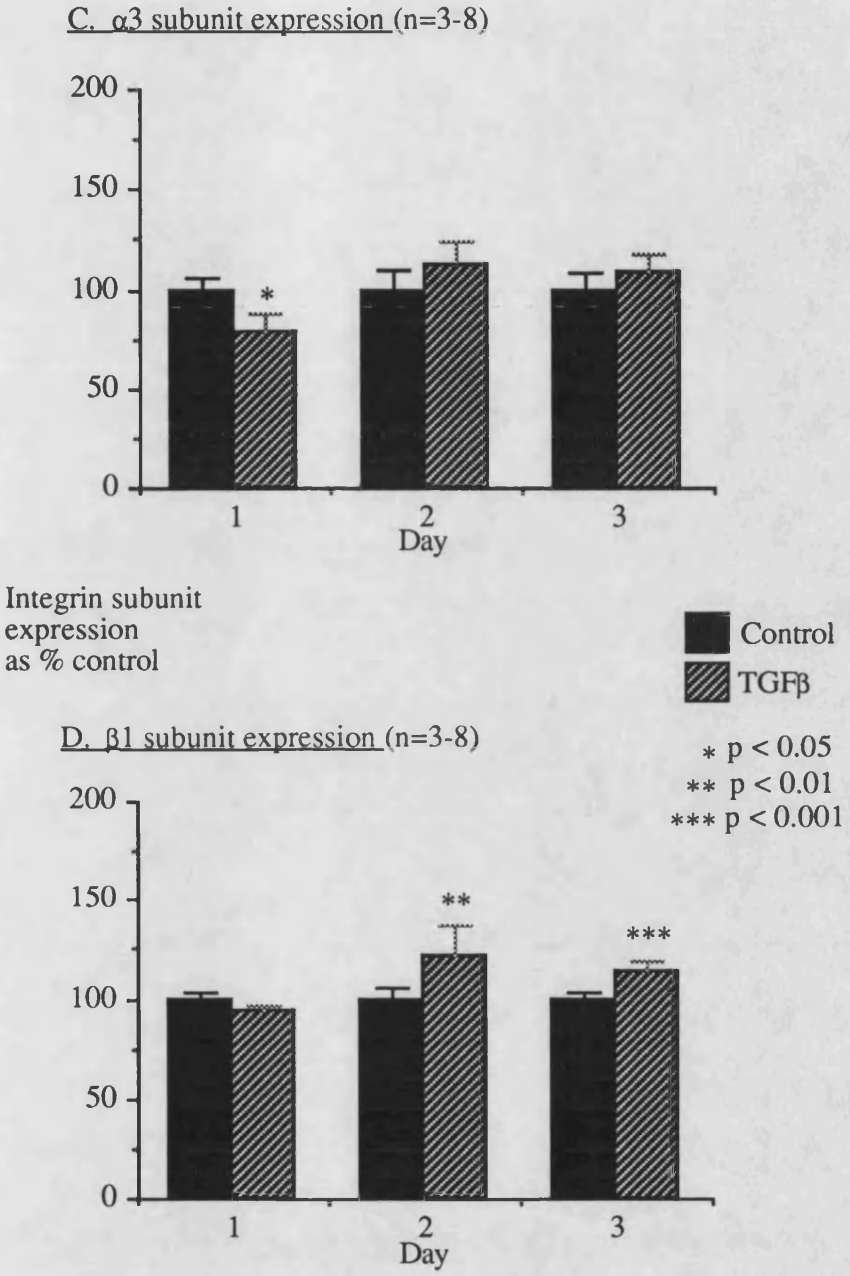


Figure 5.8. Modulation of integrin subunit expression by TGF β.

Human osteoblast-like cells were treated with TGF β (25 ng/ml) for 24, 48 and 72 hr and then prepared for FACS analysis using saturating concentrations of mAb (or IgG). MFI values were converted to receptor sites/cell and integrin subunit expression was represented as a % of control values i.e. the no.integrin subunits expressed by control cells at each time period. Assays were undertaken in duplicate or triplicate and experiments were performed 2 or 3 times. All data were pooled and the significance of any modulation in expression of α1(A), α2(B), α3 (C) and β1 (D) subunits was assessed using an unpaired Student's T-test.

Fig. 5.9A

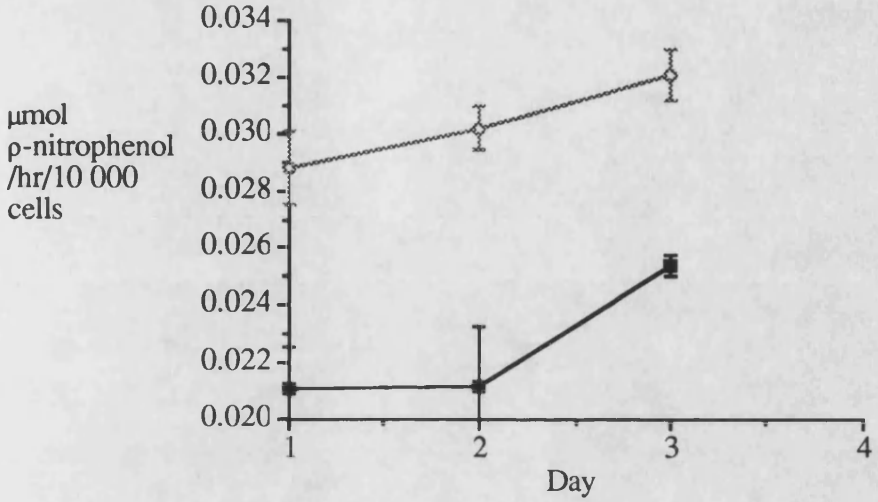


Fig. 5.9B

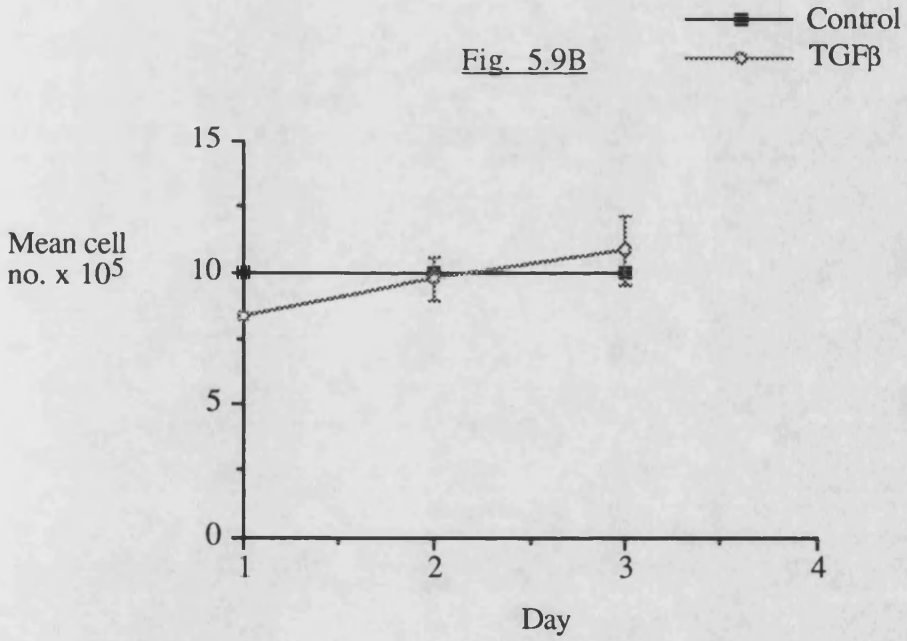
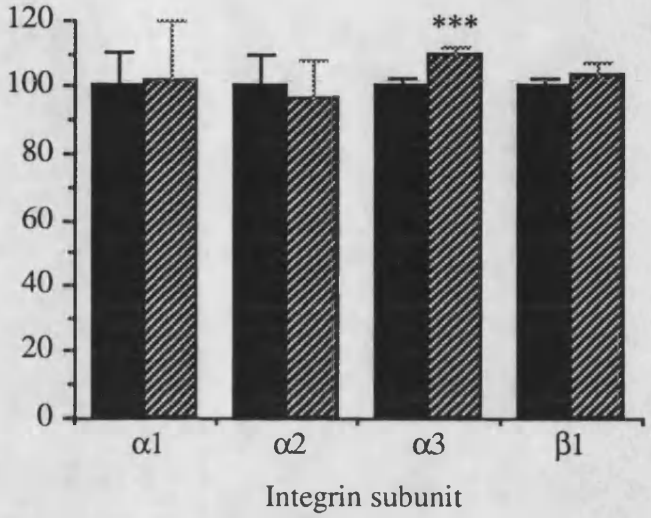


Figure 5.9 Effect of TGFβ on alkaline phospahatase activity and proliferation.

Human osteoblast-like cells were treated with TGF β (25 ng/ml) for 24-72 hr. At intervals of 24 hr, cells were assayed for alkaline phosphatase activity (A) and proliferation (B). Points are means and standard deviations from three samples and are representative of 3 experiments.

Figure 5.10

A. Growth on fibronectin (n=6)

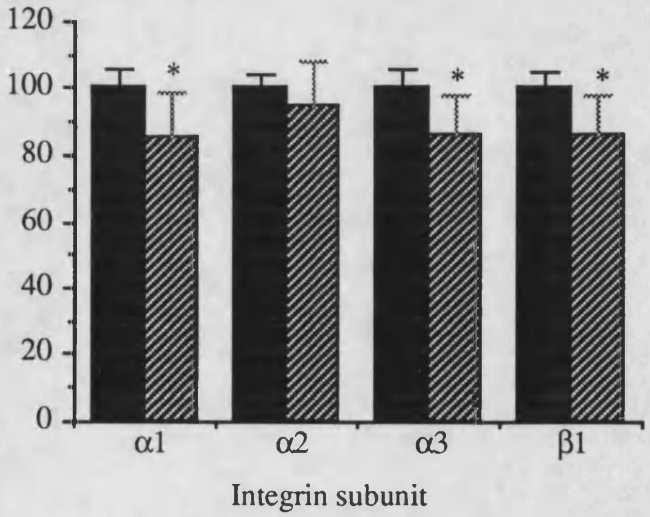


Integrin subunit expression as % control

Plastic
ECM

* $p < 0.05$
*** $p < 0.001$

B. Growth on laminin (n=6)



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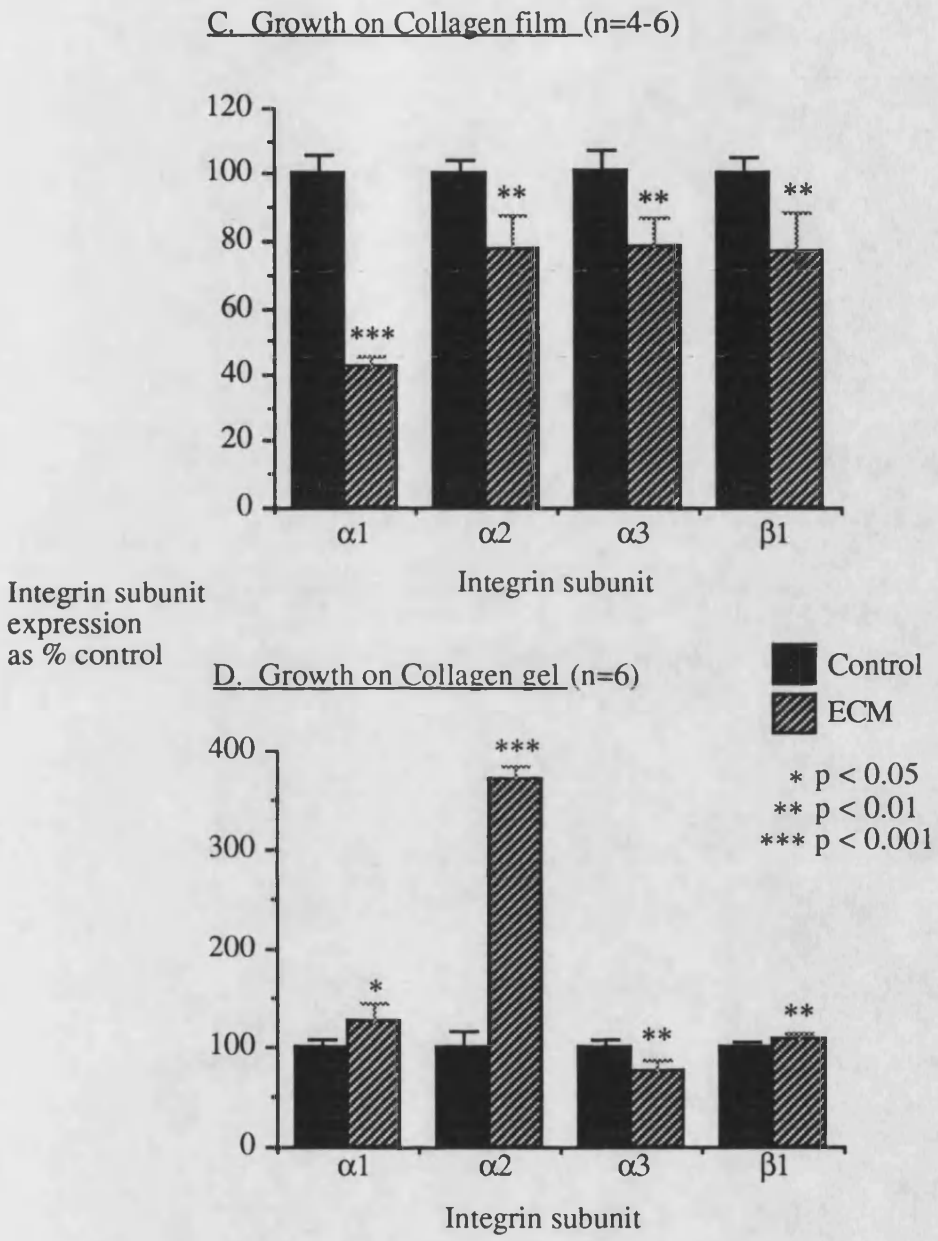


Figure 5.10 Modulation of integrin subunit expression by ECM components.

Human osteoblast-like cells were cultured on plastic (control), fibronectin (5 μg/cm^2) (A), laminin (10 μg/cm^2) (B), collagen I film (10 μg/cm^2) (C) and collagen I gel (2.5 mm) (D) for 10 days and then prepared for FACS analysis using saturating concentratrions of mAb (or IgG). MFI values were converted to receptor sites/cells and integrin subunit expression was represented as a % of control values i.e. the no.integrin subunits in cells from control plates at each time period. Assays were undertaken in triplicate and experiments were performed 2 times. All data were pooled and the significance of any modulation in expression of α1, α2, α3 and β1 subunits was assessed using an unpaired Student's T-test.

CHAPTER 6.

INTEGRIN SUBUNIT EXPRESSION BY MG-63 AND HOS-TE85 OSTEOSARCOMA CELL LINES

6.1 Abstract

The aim of this study was to select an osteosarcoma cell line whose integrin subunit profile resembled that of osteoblast-like cells and whose expression was modulated by cytokines in a similar manner to that observed in bone-derived cells. Therefore, we have assessed the integrin subunit profiles of MG-63 and HOS TE85 cells by FACS analysis and compared results with those obtained for human osteoblast-like cells. The most suitable cell line has also been treated with cytokines to determine whether integrin subunit expression was modulated in a similar fashion to bone-derived cells.

MG-63 and HOS TE85 cells expressed all the integrin subunits detected on human osteoblast-like cells. However, neither cell line exhibited an integrin subunit profile identical to that of bone cell cultures due to expression of α4 and α6 subunits by osteosarcoma cells and because of differences in the relative concentrations of individual integrin subunits expressed. MG-63 cells were considered to be more representative of bone cell cultures than HOS cells and furthermore, MG-63 cells provided good single cell suspensions for FACS analysis. Addition of IL-1β and TGFβ to cultures of MG-63 cells differentially regulated integrin subunit expression and some of these modulations in subunit expression resembled those observed in bone-derived cells. These results therefore support the use of MG-63 cells to explore the functional implications of a modulation of integrin subunit expression.

6.2 Introduction

Previous experiments investigating the role of the ECM and its corresponding cell surface receptors in regulation of bone cell phenotype have been performed using human osteoblast-like cells derived from explants of trabecular bone. These cells exhibited a doubling time of 4-5 days, and a 4-6 week growth period was required before confluency was achieved. The slow growth rate and the difficulty in obtaining sufficient bone for establishment of cultures made assays difficult to develop and repetitive experiments difficult to perform. Osteosarcoma cell lines could provide an alternative source of osteoblast-like cells. These cells divide more rapidly than primary cultures and can be maintained by routine subculture. However, these cells possess abnormal growth characteristics and it is not always possible to extrapolate effects on homogeneous cell lines to those of bone-derived cells which contain heterogeneous cell populations comprised of cells at several stages of maturation. Therefore, if osteosarcoma cell lines are to produce data of physiological relevance, it is important that different cell lines are extensively characterised and the cell line whose particular function most closely resembles that exhibited by human osteoblast-like cells is chosen for investigation.

Two readily available human osteosarcoma cell lines are MG-63 and HOS TE85. MG-63 cells were originally derived from an osteogenic sarcoma from a 14 year old male (Heremans et al.1978) and HOS TE85 cells were from a sarcoma of a 13 year old female (McAllister et al.1971). MG-63 cells are considered to show a number of features typical of an undifferentiated osteoblast phenotype. These include the synthesis of collagen types I and III, and low basal expression of alkaline phosphatase and osteocalcin which can be induced by 1,25D (Franceschi and Young, 1990; Lajeunesse et al.1990). These cells have been used as an experimental model to study a variety of different osteoblast functions such as adhesion (Dedhar et al.1987; Franceschi et al.1987; Heino and Massague, 1989), extracellular matrix synthesis (Bassols and Massague, 1988; Franceschi et al.1988), alkaline phosphatase activity (Franceschi et al.1985; Boyan et al.1989; Franceschi and Young, 1990) and osteocalcin production (Lajeunesse et al.1990; Lajeunesse et al.1991). HOS cells have not been as widely used as MG-63 cells despite their higher basal expression of alkaline phosphatase. These cells have been previously employed for studying the effects of oestrogen (Komm et al.1988) and for the localisation of oestrogen and androgen receptors (Komm et al.1988; Benz et al.1991b). Other experiments concerning the isolation and purification of insulin-like growth factor (IGF) binding proteins

(Hassager et al.1991; Lempert et al.1991; Mohan and Baylink, 1991) have also been performed.

The aim of this study was to select an osteosarcoma cell line whose integrin subunit profile resembled that of bone cell cultures and whose expression was modulated by cytokines in a similar manner to that observed in osteoblast-like cells.

6.3 Results

6.3.1 Detection of integrin subunits on MG-63 and HOS cells by FACS analysis

Cultures of MG-63 and HOS cells were prepared for FACS analysis (Method 2.6) using all the anti-integrin mAbs to determine which integrin subunits were expressed by these cell types: mAbs derived from ascites were diluted 1/25, 1/50 and 1/100 and mAbs derived from tissue culture supernatants were added directly onto cells without prior dilution; all experiments were performed in the presence of appropriately diluted negative controls (IgG). When analysing FACS results, a dot plot of SSC against FSC was produced from negative control data, and a region enclosing the majority of the population was selected and used for subsequent analysis (Fig. 6.1). Histograms for individual integrin subunits, of cell number against fluorescence, were overlayed with data obtained from negative controls and the integrin subunit expression of both osteosarcoma cell lines was determined (data not shown). MG-63 and HOS cells exhibited a very similar profile of integrin subunits. $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αV and $\beta 1$ could be detected on both cell types and there was no staining with mAbs directed against $\beta 2$ and $\beta 3$. Except for the expression of $\alpha 4$ and $\alpha 6$ subunits, this pattern of integrin subunit expression was qualitatively similar to that observed for human osteoblast-like cells (Table 6.2).

6.3.2 Titration of mAbs for antigenic detection

Accurate quantitation of the number of integrin receptor sites/cell, requires that mAbs are present at saturating concentrations. Therefore MG-63 and HOS cells were prepared for FACS analysis (Method 2.6) using all the positive anti-integrin mAbs (or IgG) over a range of mAb concentrations. No such titration experiments were therefore performed with mAbs raised against $\alpha 5$ and αV; the optimal dilutions for these mAbs were 1/25 and neat respectively. When analysing FACS data, MFI values were obtained for each sample and titration curves of fluorescence against mAb dilution were

prepared. Optimal mAb dilutions obtained for both osteosarcoma cell lines were identical and are summarised in Table 6.1.

Table 6.1. mAb dilutions for FACS experiments using osteosarcoma cell lines

Integrin subunit detected	mAb	Optimal dilution of mAb
α1	T52/7	1/100
α2	G19	1/100
α3	PB15	1/50
α4	HP2/1	1/25
α5	SAM1	1/25
α6	GoH3	1/50
αV	23C6	neat
β1	4B4	1/50

MG-63 and HOS cells were cultured for 72 hr and prepared for FACS analysis using anti-integrin mAbs (or equivalent concentrations of IgG) (Method 2.6). mAbs derived from ascites were diluted between 1/25 and 1/3200 and mAbs from tissue cultures supernatants were added directly onto cells without prior dilution. MFI values were used to plot a titration curve and optimum concentrations of anti-integrin mAbs were determined.

6.3.3 Quantitation of integrin subunit expression on MG-63 and HOS cells

MG-63 and HOS cells were cultured for 72 hr and then prepared for flow cytometry using saturating concentrations of mAb or IgG (Method 2.6). MFI values obtained for each of the integrin subunits were converted to receptor sites/cell and compared with previous data obtained from bone cell cultures. MG-63 and HOS cells expressed all the integrin subunits detected on human osteoblast-like cells and in each cell type, α3 and β1 were the subunits most highly expressed (Table 6.2). However, compared to MG-63 and HOS cells, normal bone-derived cells expressed greater numbers of α1, α2 and α5 subunits and lesser numbers of α3 subunits.

The aim of this study was to select an osteosarcoma cell line whose integrin subunit profile resembled that of human osteoblast-like cells. The major subunits detected on bone cell cultures were α1, α2, α3 and β1. Both osteosarcoma cell lines strongly

Table 6.2 A comparison of integrin subunit expression by MG-63, HOS and human osteoblast-like cells

	Integrin subunit sites/cell		
Integrin subunit detected	MG-63 cells	HOS cells	Human osteoblast -like cells
α1	18407 (767)	50859 (6067)	101023 (6077)
α2	65730 (2434)	6675 (775)	101866 (6707)
α3	276055 (6900)	255905 (20306)	127184 (5134)
α4	35652 (574)	20048 (821)	negative
α5	19428 (771)	27147 (4196)	47544 (3735)
α6	21091 (1272)	31097 (2537)	negative
αV	13822 (240)	25793 (2045)	15561 (1701)
β1	573787 (21878)	386258 (29561)	556947 (26167)
β2	negative	negative	negative
β3	negative	negative	negative

MG-63, HOS and human osteoblast-like cells were cultured for 72 hr and prepared for FACS analysis using saturating concentrations of mAb (or IgG) (Method 2.6). MFI values were converted to receptor sites/cell and data obtained from a minimum of 9 different samples were pooled to produce means and standard errors for each of the integrin subunits.

expressed α3 and β1 but the expression of α1 and α2 was dependent on cell type. Compared to HOS cells, MG-63 cells expressed greater numbers of α2 subunits but lesser numbers of α1 subunits. Overall however, it was judged that the integrin subunit profile of MG-63 cells more closely approximated to that of normal bone-derived cells. The differentiated response of MG-63 cells observed following addition of 1,25D also closely resembled that of normal osteoblast-like cells (Franceschi and Young, 1990; Lajeunesse et al.1990) and therefore this osteosarcoma cell line was selected for further investigation.

6.3.4 Modulation of integrin subunit expression on MG-63 cells by IL-1β and TGFβ

Confluent cultures of MG-63 cells were treated with test agents for 24-72 hr (Method 2.4.6) and prepared for FACS analysis using saturating concentrations of mAb (or IgG) (Method 2.6). The effects of IL-1β (10 U/ml) and TGFβ (25 ng/ml) on expression of α1, α2, α3 and β1 subunits are illustrated in Figures 6.2 and 6.3 respectively. In control cultures, over the course of these experiments, there was no significant variation in the numbers of α1, α2, α3 and β1 subunits expressed. The addition of IL-1β significantly increased the expression of α1 (Fig. 6.2A) and α2 subunits (Fig. 6.2B) at all time points. In contrast, there was a decreased expression of α3 (Fig. 6.2C) over a similar time period and a reduction in levels of β1 (Fig. 6.2D) after 72 hr. In the presence of TGFβ there was a time dependent increase in the expression of α2 (Fig. 6.3B) subunits over a period of 24-72 hr. α1 (Fig. 6.3B) and β1 (Fig. 6.3D) subunits were also significantly increased but the effects were not as pronounced as those observed for α2. In contrast, there was a significant decrease in α3 (Fig. 6.3C) subunit expression which was first detectable after 48 hr.

6.4 Discussion

Integrin subunit profiles of MG-63, HOS and human osteoblast-like cells were similar in that α1, α2, α3, α5, αV and β1 subunits were identified on all cell types. In each case, α3 and β1 were the most highly expressed subunits which can combine to form α3β1, a receptor commonly found in cells from most tissues which binds collagen, fibronectin and laminin (Humphries, 1990). The most striking difference in subunit expression was observed with α4 and α6 subunits which were detected on MG-63 and HOS cells but not on bone-derived cells. Expression of these subunits by osteosarcoma cell lines could result from an adaptation to long term culture conditions and may not have originally been detected on these cells. However, invasion of tumour

cells requires attachment to components of the basement membrane, degradation of the basement membrane and finally, migration to the underlying stroma. The laminin receptor α6β1 has previously been shown to be over-expressed in highly invasive cells (Dedhar and Saulnier, 1990) and α4β1 is clearly associated with migration (Chan et al.1992). Therefore expression of α4 and α6 by MG-63 and HOS cells could reflect the transformed nature of these cell lines. Another difference between osteosarcoma cell lines and osteoblast-like cells concerned the relative concentrations of individual α subunits. For example, α1, α2 and α5 subunits were detected at higher levels in bone-cell cultures and α3 subunits were more strongly expressed by both osteosarcoma cell lines. A similar modulation of integrin subunit expression following oncogenic transformation has been reported elsewhere. For example, Plantefaber and Hynes, (1989) demonstrated a reduction in expression of three different α subunits with oncogenic transformation, whilst expression of α3 subunits was slightly increased or unchanged.

These comparative studies showed that MG-63 and HOS cells expressed all the integrin subunits detected on bone-derived cells. However neither cell line exhibited an integrin subunit profile identical to that observed in bone cell cultures, due to expression of α4 and α6 subunits by osteosarcoma cell lines, and because of differences in relative concentrations of individual integrin subunits expressed. MG-63 cells provided better single cell suspensions than HOS cells for FACS analysis and furthermore, overall values obtained for integrin subunit sites/cell in MG-63 cells were more representative of osteoblast-like cells. Therefore experiments were performed using MG-63 cells to assess the modulation of integrin subunit expression by cytokines. Addition of IL-1β exerted significant effects on the expression of α1, α2, α3 and β1 subunits. The increased expression of α1 and α2 and the reduction in levels of α3 which was observed at 48 and 72 hr correlate well with findings observed using bone cell cultures (see Chapter 5). In addition, the elevated expression of α2 agrees with previous studies performed by Milam et al.(1991). The main difference in the modulation of integrin subunit expression between IL-1β treated MG-63 cells and osteoblast-like cells concerned expression of β1. This subunit was elevated in IL-1β treated bone cells and has previously shown to be increased in MG-63 cells (Dedhar, 1989), but in these experiments, expression of β1 was significantly reduced.

Treatment of MG-63 cells with TGFβ also modulated the expression of α1, α2, α3 and β1 subunits. The increased expression of α2 and β1 and the decreased expression of α3 correlates well with previous findings (Heino and Massague, 1989). The time dependent increase in expression of α2 and β1 subunits also agrees with results

obtained from osteoblast-like cells (see Chapter 5). However, the reduced expression of α3 and elevated expression of α1 observed in these experiments do not correspond with data from bone-derived cells.

These experiments show that integrin subunit expression of MG-63 cells is differentially regulated by cytokines and that some of the changes in subunit expression resemble those observed in human osteoblast-like cells. Having investigated the effects of IL-1β and TGFβ on the expression of α1, α2, α3 and β1 subunits, it would be interesting to examine the effects of these agents on expression of some of the other α (and β ?) subunits. It is very possible that addition of IL-1β and TGFβ would regulate the expression of other integrin subunits and evidence for this is already emerging. For example, Milam et al.(1991) showed that addition of IL-1β (10 pM; 48 hr) to cultures of MG-63 cells increased α5 and αV but decreased α4 subunit mRNA, in addition to increasing expression of α2. Heino and Massague (1989) demonstrated that addition of TGFβ (200 pM (approx.5 ng/ml); 12-24 hr in serum free medium) to MG-63 cells increased expression of α5 in addition to regulating expression of α2, α3 and β1. Finally, Ignotz et al.(1989) reported increased synthesis of β3 subunits following TGFβ administration to MG-63 cells (250 pM (approx. 6 ng/ml); 12 hr in MEM + 0.1% FCS). This latter report was rather surprising as β3 could not be detected in this cell type under our experimental conditions. Possible implications of a modulation in the expression of other integrin subunits will be discussed later (Chapter 7).

Figure 6.1A.

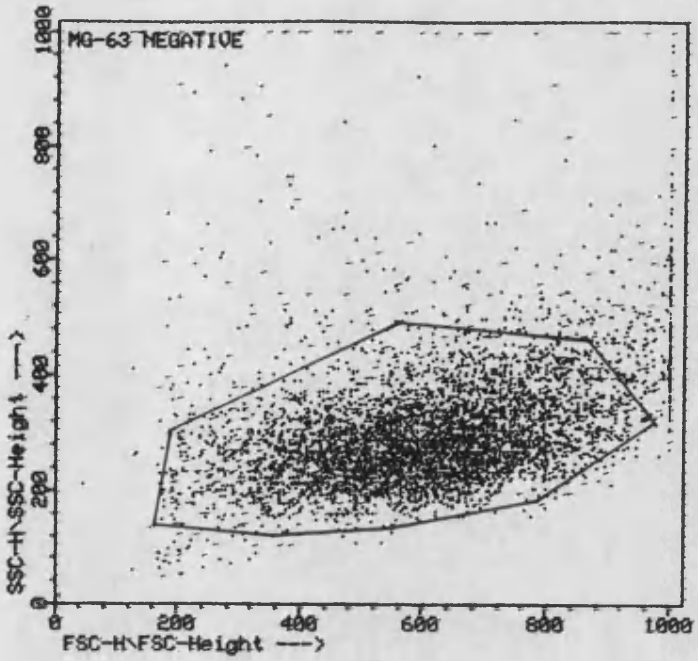


Figure 6.1B

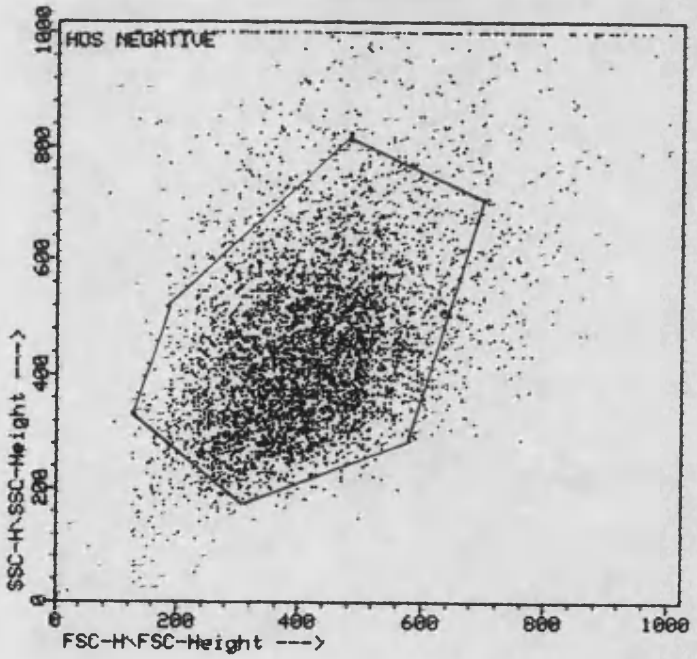
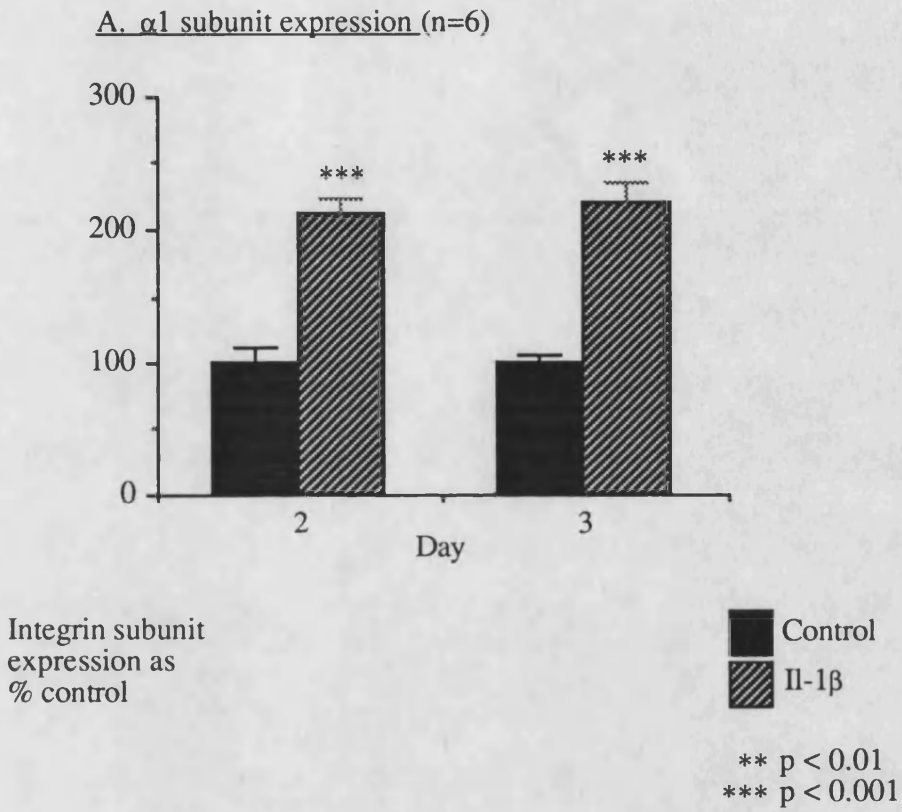


Figure 6.1 Typical populations of MG-63 (A) and HOS TE85 cells (B) prepared for FACS analysis.

Dotplots of side scatter (SSC) against forward scatter (FSC) were produced from negative controls to assess the homogeneity of the cell population. Heterogeneous cells were excluded by selecting a region enclosing the majority of the population, and this was used for subsequent analysis.

Figure 6.2



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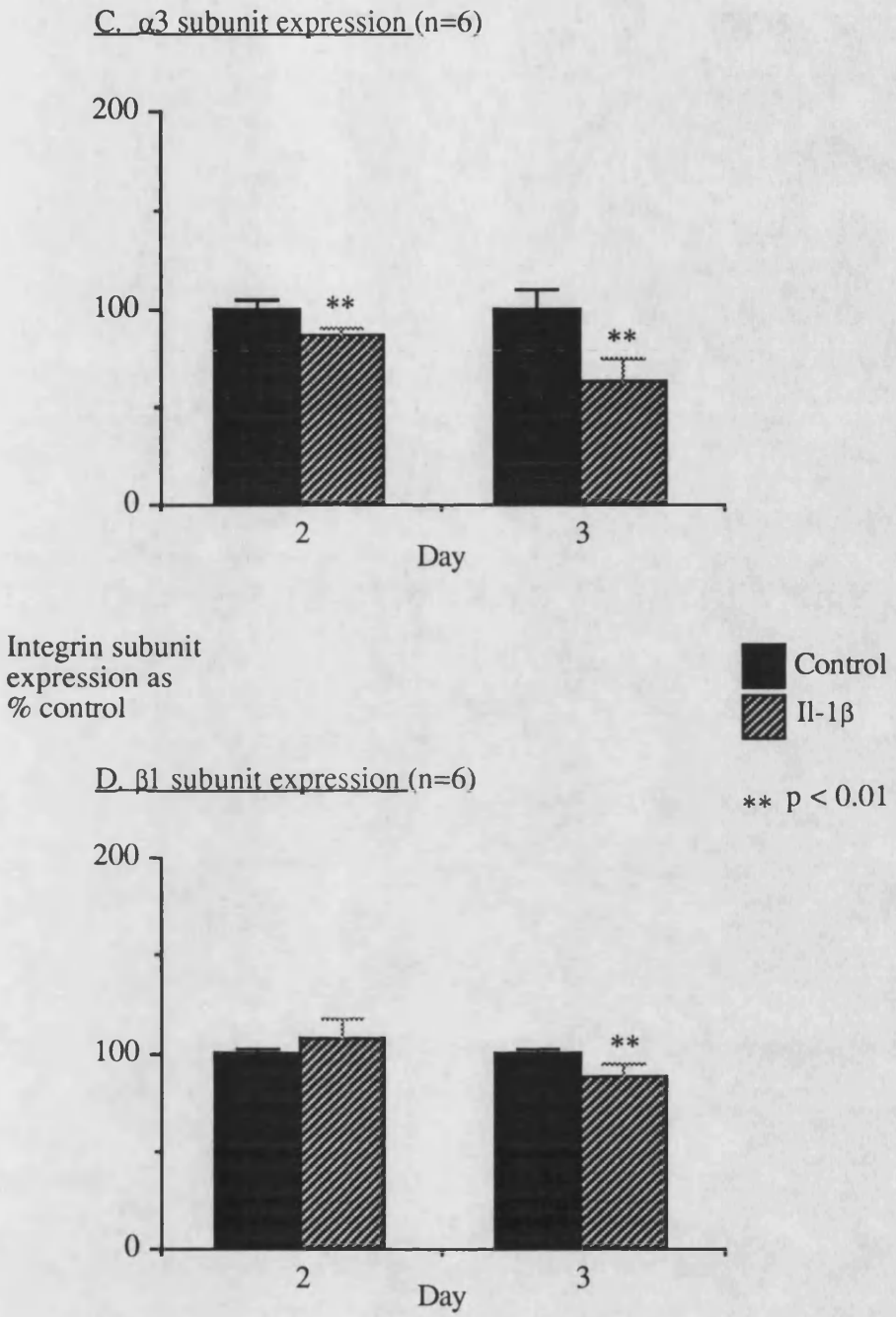
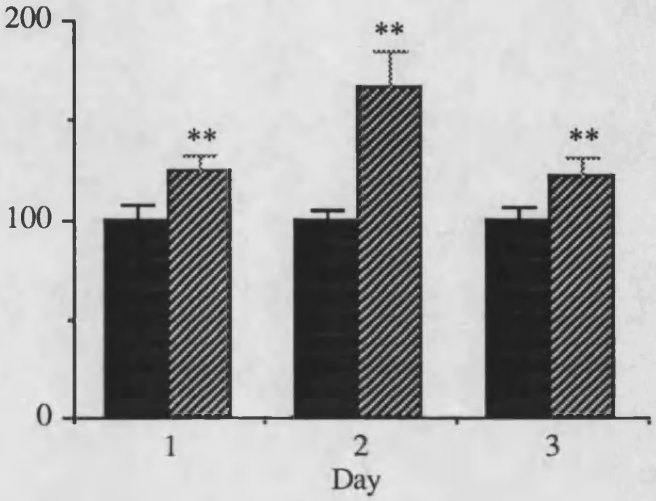


Figure 6.2. Modulation of integrin subunit expression by IL-1 β.

MG-63 cells were treated with IL-1 β (10 U/ml) for 48 and72 hr and prepared for FACS analysis using saturating concentrations of mAb (or IgG). MFI values were converted to receptor sites/cell and expression of α1 (A), α2 (B), α3 (C) and β1 (D) subunits was expressed as a % of control values i.e the no. of α1, α2, α3 and β1 subunits expressed by cells in control plates at each time period. Assays were undertaken in triplicate and experiments were performed twice. Values represent means and standard deviations from pooled data: the significance of any modulation of expression was assessed using an unpaired Student's T-test

Figure 6.3

A. α1 subunit expression (n=6)

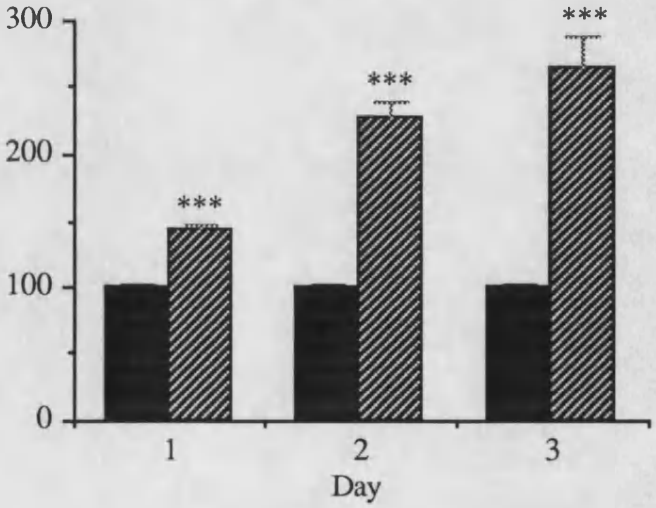


Integrin subunit expression as % control

Control
TGFβ

** p < 0.01
*** p < 0.001

B. α2 subunit expression (n=6)



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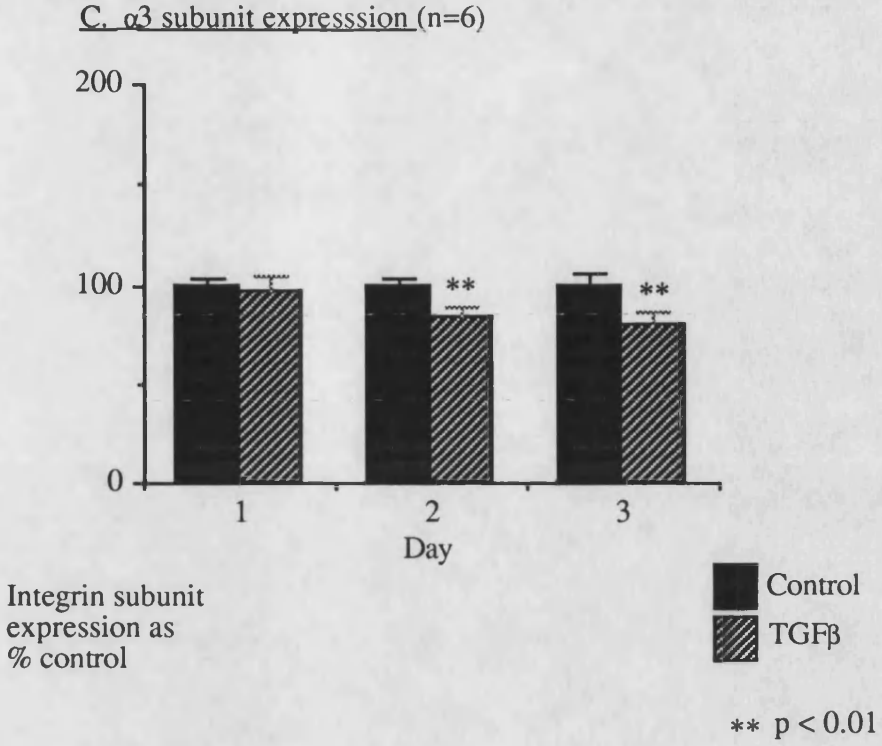


Figure 6.3. Modulation of integrin subunit expression by TGF β.

MG-63 cells were treated with TGF β (25 ng/ml) for 24-72 hr and prepared for FACS analysis using saturating concentrations of mAb (or IgG). MFI values were converted to receptor sites/cell and expression of α1 (A), α2 (B), α3 (C) and β1 (D) subunits was expressed as a % of control values i.e the no. of α1, α2, α3 and β1 subunits expressed by cells in control plates at each time period. Assays were undertaken in triplicate and experiments were performed twice. Values represent means and standard deviations from pooled data: the significance of any modulation of expression was assessed using an unpaired Student's T-test

CHAPTER 7.

THE EFFECT OF CYTOKINES ON ADHESION OF MG-63 CELLS TO COLLAGEN I, FIBRONECTIN AND LAMININ

7.1 Abstract

Previous studies demonstrated that the integrin subunit expression of human osteoblast-like cells and MG-63 cells was modulated by IL-1β and TGFβ. To assess the functional significance of these findings, an assay was developed using MG-63 cells to determine the effects of IL-1β and TGFβ on cellular adhesion. Studies were performed using dishes coated with collagen I, fibronectin and laminin since it could be inferred from the integrin subunit profiles of MG-63 cells (and osteoblast-like cells) that these proteins were likely adhesion substrates. Cell adherence was assessed by protein assay which was found to be more sensitive than MTT and more convenient than measuring ^{3}H-thymidine incorporation. Background ECM proteins and blocking reagents did not interfere with the absorbance readings obtained in this assay.

Under basal conditions, maximal adhesion to collagen I and fibronectin occurred at 0.1 μg/cm^2 whereas maximal adhesion to laminin occurred at 5-20 μg/cm^2. At these concentrations, 90-100 % of cells attached to all matrices, compared with 20-40 % in uncoated wells. Time course experiments demonstrated that cells attached to these ECM proteins at similar rates, maximal adhesion occurring after 2 hr. Pre-treatment of MG-63 cells with IL-1β for 72 hr did not affect adhesion to uncoated plastic, collagen I or fibronectin over a concentration range of 0.001-5 μg/cm^2. However, adhesion to some concentrations of laminin was significantly reduced (total concentration range 0.01-20 μg/cm^2). Addition of TGFβ for a similar time period did not affect adhesion to plastic or any of the other ECM proteins tested.

7.2 Introduction

The process of bone remodelling consists of a complex sequence of cellular events occurring at discrete sites in the skeleton. It must be temporally and spatially controlled to ensure that the amount of bone resorption balances the amount of bone formation. Cytokines are among the local mediators considered to play an important regulatory role because they are extremely potent and labile and can affect many different aspects of cell function (Gowen, 1991; Gowen, 1992b; MacDonald and Gowen, 1992). As over 90% of bone is composed of matrix, it seems likely that integrin-mediated adhesive interactions will also exert some kind of regulatory control. Previous experiments demonstrated that integrin subunit expression of human osteoblast-like cells was modulated by cytokines and ECM components, but the significance of this finding in terms of differentiated cell phenotype was unknown (Chapter 5). To further assess the functional relevance of this finding, an assay was developed to determine the effects of these cytokines on cell adhesion.

These experiments required very large numbers of cells and so it was decided to use an osteosarcoma cell line. MG-63 cells expressed all the integrin subunits detected on osteoblast-like cells and furthermore, integrin subunit expression was differentially regulated by IL-1β and TGFβ in a similar manner to that of bone-derived cells (Chapter 6). Integrin subunit profiles exhibited by this osteosarcoma cell line (and human osteoblast-like cells) suggested that potential ligands used for attachment include collagen I, fibronectin and laminin. Therefore, an adhesion assay was developed and the effects of IL-1β and TGFβ on % adhesion of MG-63 cells to these three ECM components were assessed.

7.3 Methods

7.3.1 A comparison of three different methods for assessment of cell number

Three commonly used techniques for the assessment of cell number are MTT reduction, ^{3}H-thymidine incorporation and protein content, using a dye binding assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a tetrazolium salt which labels metabolically active cells and can be measured colourimetrically (Mosmann, 1983). Cell protein content can be assessed colourimetrically using a Bradford protein assay which employs the stain Coomassie brilliant blue (Bradford, 1976).

To determine the effects of cytokines on cell adhesion, a sensitive assay system was required for accurately monitoring small changes in cell number. Therefore, confluent cultures of MG-63 cells were seeded into flat bottomed 96 well plates in MEM+10% FCS at a cell density of 100-20 000 cells/well (100 µl/well) and the sensitivity of these three different assay systems was compared.

7.3.1.1 MTT reduction

MG-63 cells were cultured for 24 hr at 37°C and then 20 µl/well MTT solution (5 mg/ml in PBS) was added. After 4 hr at 37°C, the medium was removed and cells were solubilised by the addition of 100 µl/well acidified isopropanol (1 ml 4 M HCl + 99 ml isopropanol). After 30 min at 37°C, the absorbance was measured on an ELISA plate reader at 630 nm and 570 nm reference and test wavelengths, respectively.

7.3.1.2 ^{3}H-thymidine incorporation

MG-63 cells were cultrued for 8 hr and then 1uCi/well ^{3}H-thymidine was added (10 µl/well). After 20 hr at 37°C, the medium was removed and cells were lysed by the addition of 20 µl/well 0.1% Tween 20. After 20 min at room temperature, 200 µl/well H_20 was added and cell lysates were transferred onto filters using a cell harvester. Wells were washed 3 times in H_20 and all insoluble contents were collected onto the filters. Filters were then transferred into scintillation vials, scintillant was added (500 µl/tube) and cell number was assessed by counting ^{3}H using a β counter.

7.3.1.3 Bradford protein assay

MG-63 cells cultured for 28 hr and then the medium was removed, cells were washed in PBS (3 x 100 µl) and lysed by the addition of 200 µl/well H_20. After 20 min at room temperature, 50 µl/well protein dye binding reagent was added (Biorad), well contents were mixed thoroughly and after a further 5 min, the absorbance was recorded at 630 nm.

7.3.2 Effect of background protein on Bradford assay

The attachment assay to be developed for these experiments was based on protocols described by Adams and Watt (1990; 1991) where cells were seeded onto pre-coated wells and attachment to ECM proteins was assessed after blocking non specific binding

using BSA. If a protein assay was to be used as a measurement of cell number, it was important to assess the effects of background protein on absorbance readings. In order to determine the effects of BSA, 100 µl BSA (2 mg/ml in PBS) was added to wells of a 96 well plate. After 1 hr at room temperature, BSA was removed and wells were washed with PBS (3 x 100 µl PBS). A standard curve (0-25 µg/ml BSA in H_20) was then prepared using a stock solution of BSA (100 µg/ml) and 200 µl aliquots were transferred into control wells and wells pre-coated with BSA. To assess the effects of ECM proteins, flat bottomed 96 well plates were coated with collagen I, fibronectin or laminin (0-100 µg/cm^2 in PBS) and then BSA (Method 2.7). Wells were washed extensively to remove unbound protein (3 x 100 µl PBS) and 200 µl/well H_20 was replaced. 50 µl protein dye binding reagent was then added to all wells, the contents were mixed thoroughly and after 5 min, the absorbance was recorded at 630 nm.

7.4 Results

7.4.1 A comparison of three different methods for assessment of cell number

MG-63 cells were cultured for a total of 28 hr and then cell number was assessed by MTT reduction, ^{3}H-thymidine incorporation or by protein assay. The Bradford protein assay (Fig. 7.1A) produced a larger change in absorbance readings over a concentration of 100-20 000 cells/well than the MTT assay (Fig. 7.1B). ^{3}H-thymidine incorporation (Fig. 7.1C) also gave a good range of counts over these cell concentrations but this assay was very time consuming and in light of the number of experiments that could be performed, it was decided to use the protein assay in subsequent experiments to assess cell attachment.

7.4.2 Effect of background protein on Bradford assay

Preliminary experiments were performed to assess the effects of ECM protein and BSA on the Bradford protein assay. It can be seen from Figure 7.2 and Table 7.1 that a BSA coating did not interfere with the protein standard curve and that absorbance readings obtained from wells coated with ECM proteins were not substantially different from those of wells coated with PBS.

Table 7.1 Effect of ECM proteins on Bradford assay

Concentration ECM protein ($\mu g/cm^2$)	Fibronectin	Collagen I	Laminin
0.00	.000	.000	.000
0.10	.004	.005	-.006
0.50	.002	.009	.005
1.00	.000	.002	.000
5.00	.005	.001	.001
20.0	.000	.01	.005
50.0	.003	.018	.006
100.0	.006	.010	.005

A Bradford protein assay was performed in flat bottomed 96 well plates pre-coated with collagen I, fibronectin or laminin (0-100 $\mu g/cm^2$) and then with BSA (2 mg/ml).

7.4.3 Adhesive properties of MG-63 cells

7.4.3.1 Concentration dependent binding to selected ECM proteins

MG-63 cells were seeded into flat bottomed 96 well plates pre-coated with 0.001-100 $\mu g/cm^2$ collagen I, fibronectin or laminin and after 4 hr at 37°C, attached cell protein was assessed (Method 2.7). MG-63 cells bound in a saturable manner to all three ECM proteins (Fig. 7.3A). Adhesion to collagen I and fibronectin was very similar and maximum attachment occurred at 0.1 $\mu g/cm^2$. In contrast, cells bound to laminin less readily, with optimum attachment occurring at 5 $\mu g/cm^2$.

7.4.3.2 Time dependent binding to selected ECM proteins

MG-63 cells were seeded into flat bottomed 96 well plates pre-coated with collagen I, fibronectin (5 $\mu g/cm^2$) or laminin (20 $\mu g/cm^2$), and attached cell protein was assessed at intervals over the next 5 hr (Method 2.7). Cells attached to collagen I, fibronectin and laminin at similar rates; maximal attachment occurring after 2 hr (Fig. 7.3B).

In light of these findings, subsequent adhesion assays were performed after 2 hr using 0.001-0.5 $\mu g/cm^2$ fibronectin and collagen I, and 0.01-20 $\mu g/cm^2$ laminin.

7.4.4 % Adhesion of MG-63 cells to ECM proteins

Having optimised the assay conditions, it was decided to correct for any differences in seeding densities by expressing adhesion to ECM proteins as a percentage of the total protein/well. Therefore cells were seeded into pre-coated wells (or uncoated wells) in serum free medium as described and after 2 hr, total protein was assessed (Method 2.7). Dose response curves for ECM proteins were similar to those described previously (Fig. 7.4). Cells attached to collagen I and fibronectin very readily and 100% adhesion occurred at 0.1 $\mu g/cm^2$. A higher concentration was required for attachment to laminin and 5-20 $\mu g/cm^2$ was required for 100% adhesion. Attachment to uncoated plastic was low by comparison, and values of approximately 20% were typically obtained in these experiments.

7.4.5 Effect of cytokines on cell adhesion

Confluent cultures of MG-63 cells were cultured in the presence and absence of IL-1β and TGFβ for 72 hr (Method 2.4.6) and % adhesion to plastic and different ECM proteins was assessed (Method 2.7). Typical results from experiments investigating the effects of IL-1β and TGFβ are illustrated in Figures 7.5 and 7.6 respectively. Neither of these agents significantly affected background adhesion to plastic which accounted for 20-40% of the total adhesion. Addition of IL-1β did not affect adhesion to collagen I (Fig. 7.5A) or fibronectin (Fig. 7.5B) but adhesion to some concentrations of laminin were slightly but statistically reduced (Fig. 7.5C). In the experiment shown, adhesion to 0.1, 1.0 and 10 $\mu g/cm^2$ laminin was decreased but the concentrations at which this reduction occurred were not consistent between experiments. Treatment with TGFβ did not affect adhesion to collagen I (Fig. 7.6A) but attachment to fibronectin (Fig. 7.6B) and laminin (Fig. 7.6C) appeared to be slightly increased. However, none of these findings was statistically significant.

7.5 Discussion

The adhesive properties of MG-63 cells observed under basal conditions were similar to those described by other investigators (Heino and Massague, 1989; Santala and Heino, 1991; Brighton and Albeda, 1992). However, in previous studies, fewer cells adhered to laminin than to collagen I and fibronectin (Heino and Massague, 1989; Brighton and Albeda, 1992) and attachment to laminin occurred at a slower rate than that observed for other ECM proteins (Heino and Massague, 1989). The comparatively low values obtained for % adhesion to laminin may have resulted from differences in

experimental protocol. In all these reports, adhesion was assessed after washing attached cells three times in PBS and therefore values obtained for % cell adhesion would also depend on the strength of attachment to different matrix components. In our laboratory, attachment was assessed after one PBS wash which was undertaken carefully to prevent detachment of loosely adherent cells.

Treatment of MG-63 cells with IL-1β and TGFβ (72 hr) did not markedly affect cell adhesion. The lack of effects on attachment to collagen I and fibronectin correlate with previous studies performed using this cell type (Heino and Massague, 1989; Santala and Heino, 1991). However in these reports, IL-1β increased adhesion to laminin by 1.22-1.3 fold (Santala and Heino, 1991) and TGFβ reduced adhesion to laminin by 39-59% (Heino and Massague, 1989). These experiments were performed in serum free medium after a 12 hr treatment period and it is possible that these variations accounted for the different effects. However, the changes in integrin subunit expression reported by these authors following treatment with IL-1β and TGFβ were similar to those observed in our laboratory. If a modulation of integrin subunit expression is important for cell adhesion one would predict that the effects of cytokines on attachment would be very similar.

The minimal effects of IL-1β and TGFβ on adhesion of MG-63 cells to collagen I and fibronectin could result from ligand redundancy. MG-63 cells expressed three collagen receptors (α1β1, α2β1, α3β1) and four fibronectin receptors (α3β1, α4β1, α5β1 and αVβ1) and therefore a modulation in expression of one of these receptors could easily be masked by another integrin molecule binding to the same ligand. IL-1β and TGFβ increased expression of α1 and α2 subunits but decreased expression of α3 (Chapter 6): the fact that neither of these cytokines affected adhesion to collagen I suggests that the decrease in expression of α3 subunits was masked by the increased expression of α1 and α2. The situation is made more complex by the modulation of other α integrin subunits and differences in receptor affinity. For example, IL-1β has previously been shown to increase expression of α5 and αV, and to decrease expression of α4 subunit mRNA (Milam et al.1991). The α5β1 heterodimer is considered to be of a higher affinity nature than α3β1 (Plantefaber and Hynes, 1989) and therefore increased expression of α5 could compensate for the decreased expression of α3. This in turn could minimise the effects of Il-1β on adhesion of MG-63 cells to fibronectin.

From previous studies (Heino and Massague, 1989; Santala and Heino, 1991) it would appear that major effects of IL-1β and TGFβ on the adhesive properties of MG-63 cells occur following attachment to laminin. This suggests that even though MG-63 cells

express four potential laminin receptors (α1β1, α2β1, a3b1 and α6β1), integrin-mediated adhesion to this ECM protein is fairly specific. In our laboratory, the decreased adhesion to laminin observed following addition of IL-1β could be correlated with a decreased expression of α3 and β1 subunits. This suggests that α3β1 is the major integrin molecule responsible for mediating adhesion to laminin. The lack of significant effects of TGFβ on adhesion to laminin despite the reduction in α3 subunit expression observed following treatment with this cytokine (Chapter 6) could result from the increased expression of β1 subunits. Alternatively, these findings could result for the sensitivity of the experimental assay: addition of IL-1β for 72 hr decreased α3 subunit expression by approximately 38% whereas treatment with TGFβ for a similar time period only decreased of α3 subunit expression by approximately 21%. The smaller changes in integrin subunit expression observed following addition of TGFβ may not be sufficient to detect a change in cell adhesion using this assay.

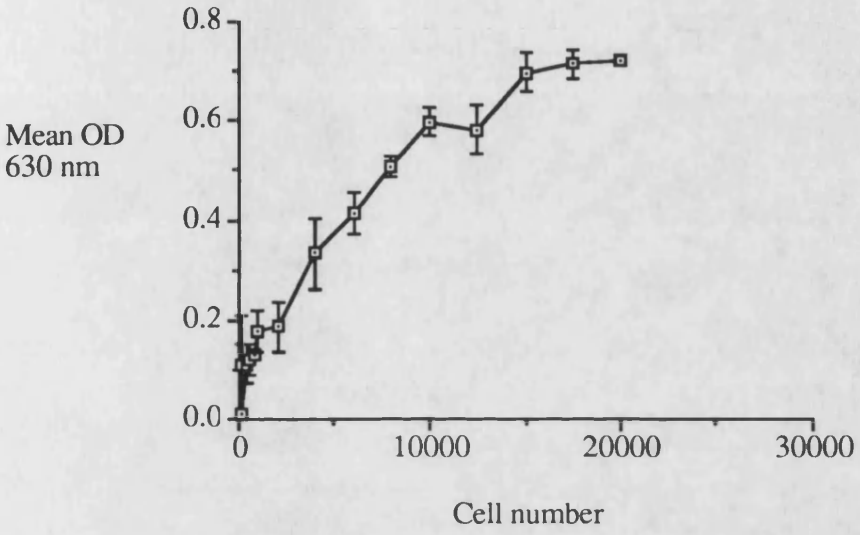
Apart from the effects of ligand redundancy and receptor affinity, there are many technical problems which, with hindsight, could be associated with the minimal responses observed in this assay. The effects of cytokines on adhesion of MG-63 cells were assessed after trypsinisation. It is possible that even though integrin subunits were immunologically 'intact' i.e. recognised by mAbs, they were not functional and therefore not contributing to cell adhesion. It would have been better to detach cells using EDTA or collagenase and then perform adhesion assays in the presence of cycloheximide to ensure that attachment resulted from existing integrin subunits. Another possibility would be to assess the functional relevance of a modulation of integrin subunit expression *in situ*. For example, the effects of cytokines could be determined by measuring the strength of attachment: one such experiment was performed (data not shown) but unfortunately there was not sufficient time to optimise the assay conditions.

Another problem associated with this adhesion assay was that non specific basal adhesion to uncoated wells accounted for 20-40% of the total adhesion and cells attached very readily to all ECM proteins over the appropriate concentration range. In order to see a difference in cell attachment properties, the difference between control and treated cells would therefore need to be very great. All these experiments were performed using 96 well plates treated for tissue culture. It would have been better to use bacteriological plastic to reduce non specific binding and to maximise absorption of ECM proteins. Another improvement could be made when determining % adhesion. It would have been better to measure non attached cell protein in each well since pooling supernatants does not correct for inter-well variations at each ECM protein

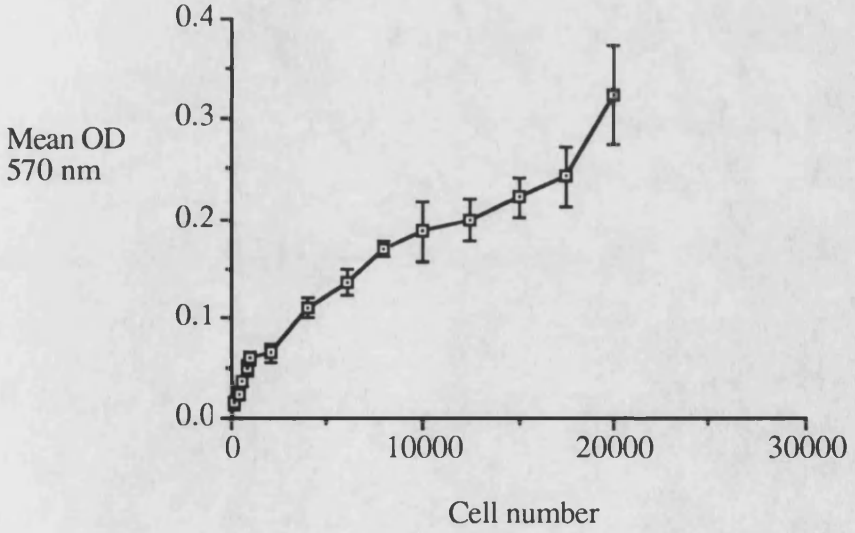
concentration. Results would perhaps also have been more meaningful if a larger number of samples had been statistically analysed (n=3): it was not possible to pool data from multiple studies due to inter-experiment variation and therefore each experiment should have contained a larger number of replicates. Finally, MG-63 cells were probably not an ideal experimental model of human osteoblast-like cells. This osteosarcoma cell line expressed α3 subunits at levels 5 fold higher than α2 and 15 fold higher than α1. In view of the low affinity nature of these cell surface receptors, a modulation in expression of α1 or α2 subunits is likely to be less relevant than a modulation in expression of α3. Human bone-derived cells expressed lower levels of α3 and relative concentrations of α1 and α2 subunits were much greater. It would therefore be interesting to repeat these experiments using this cell type to determine whether any more significant responses could be obtained.

Figure 7.1

A. Bradford protein assay



B) MTT assay



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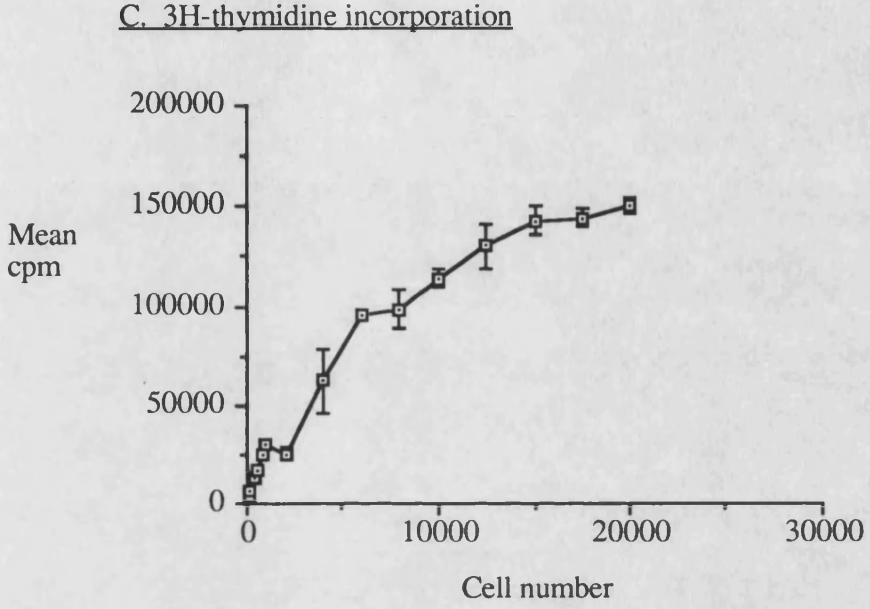


Figure 7.1 A comparison of three different assays for assessment of cell number.

MG-63 cells were seeded into flat bottomed 96 well plates in MEM+10% FCS at a cell density of 100-20 000 cells/well. After a total of 28 hr, cell number was assessed by MTT reduction (A), Bradford protein assay (B) and 3H-thymidine incorporation (C). Points represent means and standard deviations from 6 different wells.

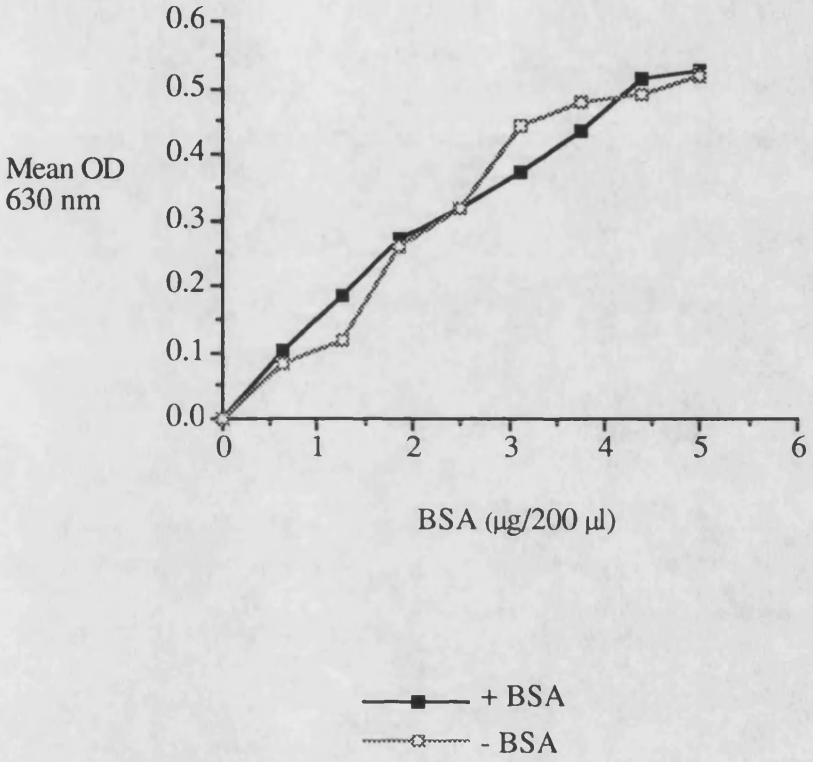


Figure 7.2 Effect of BSA on protein assay standard curve.

A protein standard curve was prepared using BSA over a concentration of 0-25 µg/ml. 4 x 200 µl aliquots were transferred to wells of a 96-well plate, half of which had been pre-coated with BSA (2 mg/ml, 1 hr). 50 µl/well dye reagent was added to all wells, the contents were mixed thoroughly and after 5 mins, the absorbance was recorded at 630 nm. Duplicates varied by < 10 %.

Fig 7.3A

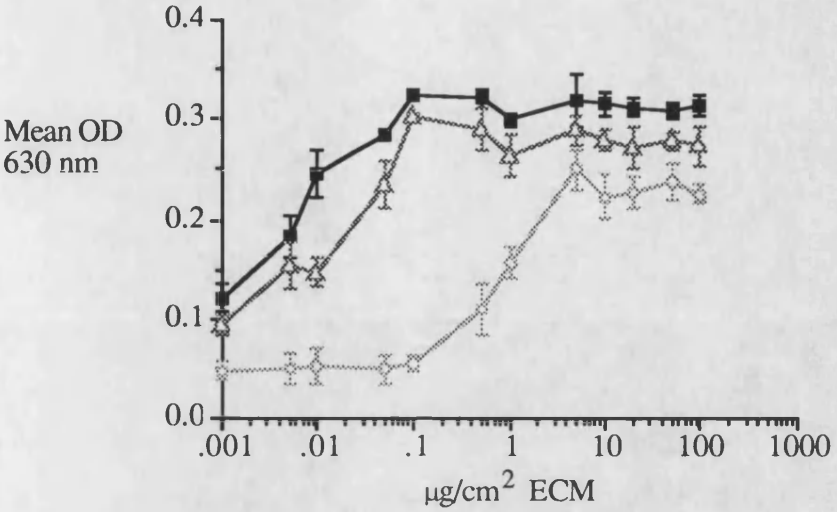


Fig 7.3B

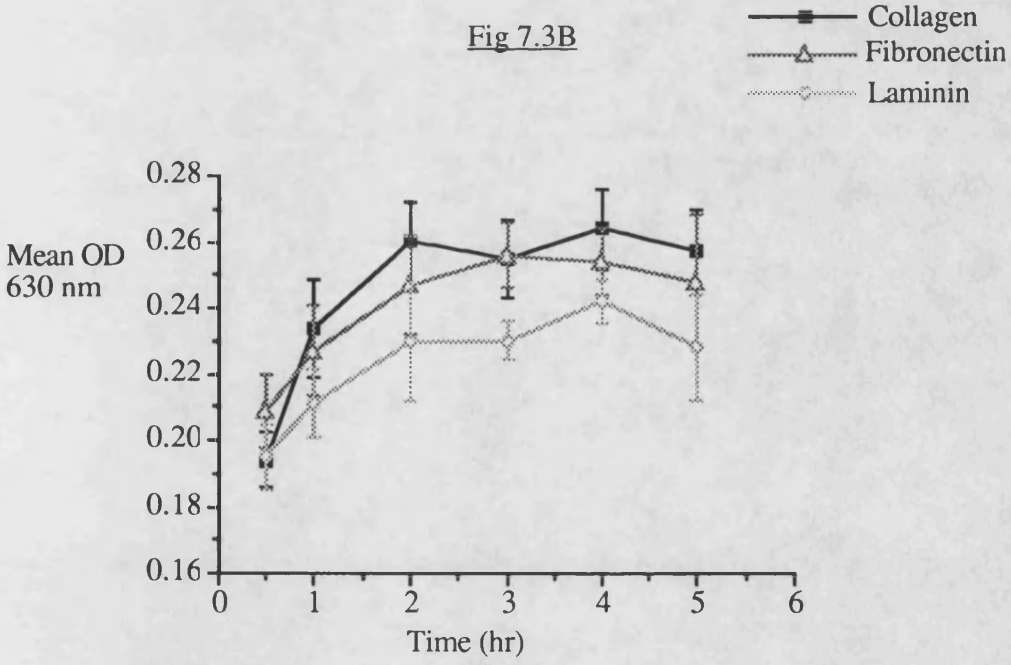


Figure 7.3 Adhesion of MG-63 cells to ECM proteins.

MG-63 cells (15 000) were seeded into microtitre wells pre-coated with collagen I, fibronectin and laminin in serum free MEM. Attachment after 4 hr at 37°C to different concentrations of ECM proteins (A). Time course for attachment to collagen I, fibronectin (5 µg/cm^2) and laminin (20 µg/cm^2) (B). Points shown are means and standard deviations from 6 wells and are representative of 3 different experiments.

Fig 7.3A

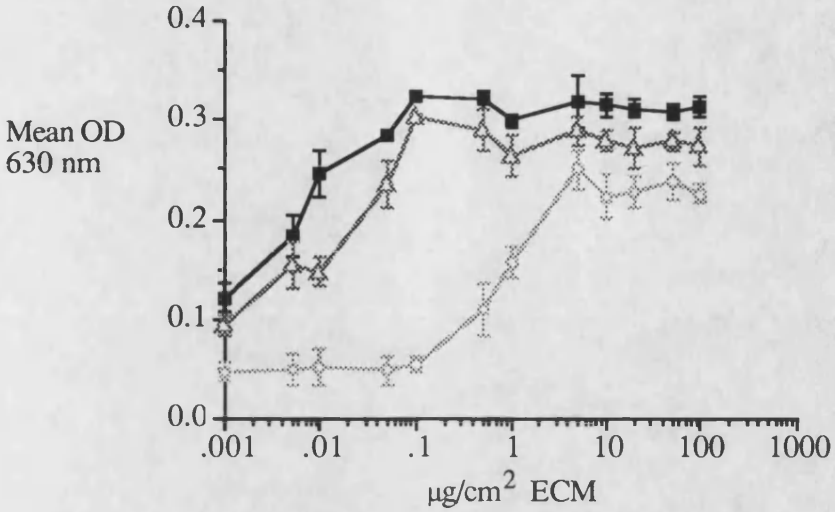


Fig 7.3B

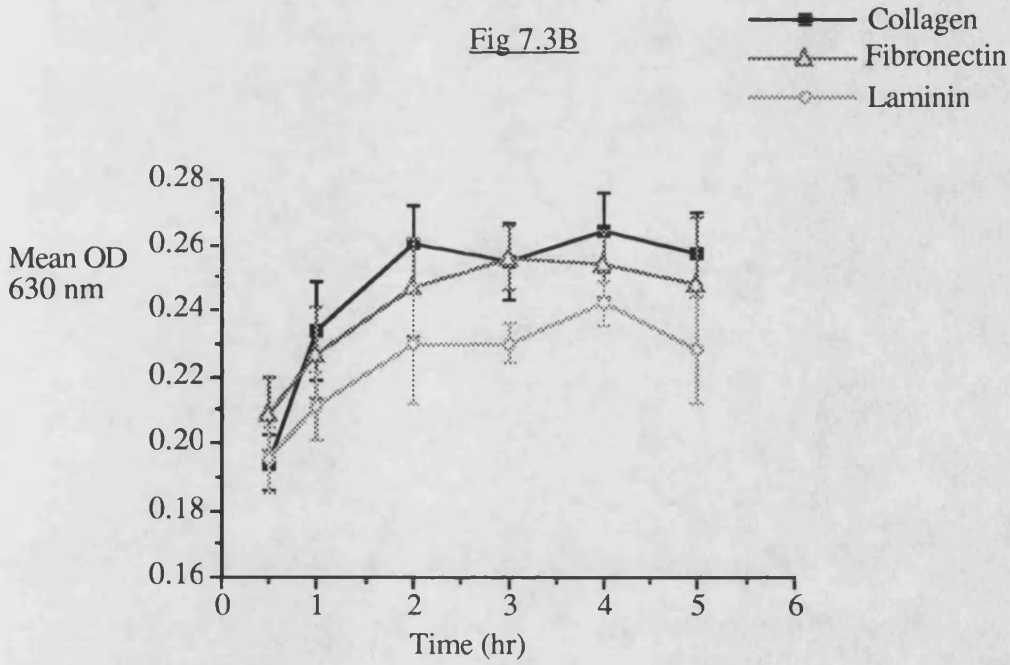
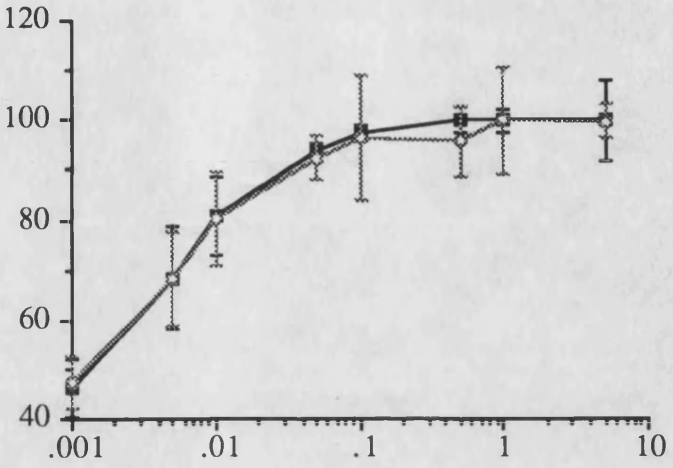


Figure 7.3 Adhesion of MG-63 cells to ECM proteins.

MG-63 cells (15 000) were seeded into microtitre wells pre-coated with collagen I, fibronectin and laminin in serum free MEM. Attachment after 4 hr at 37 °C to different concentrations of ECM proteins (A). Time course for attachment to collagen I, fibronectin (5 µg/cm^2) and laminin (20 µg/cm^2) (B). Points shown are means and standard deviations from 6 wells and are representative of 3 different experiments.

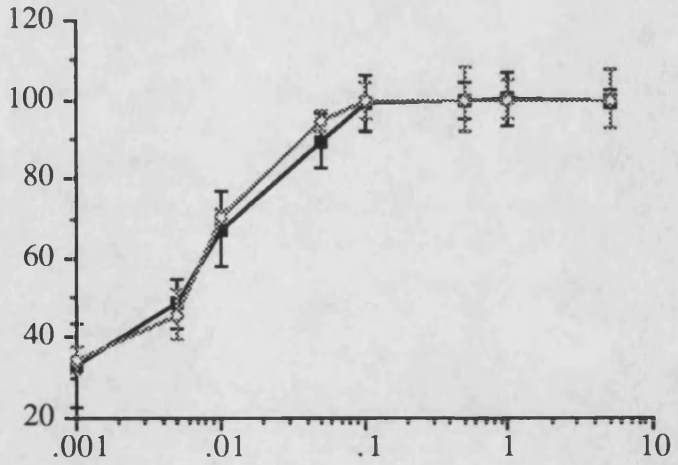
Figure 7.5

A. Collagen I



% Adhesion

B. Fibronectin



Control
Il-1β

cont...

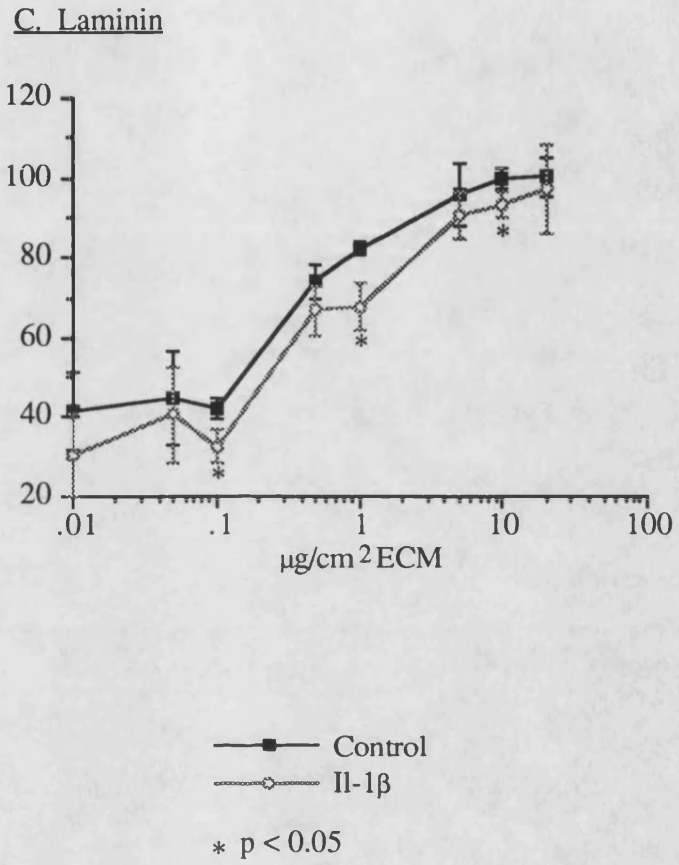
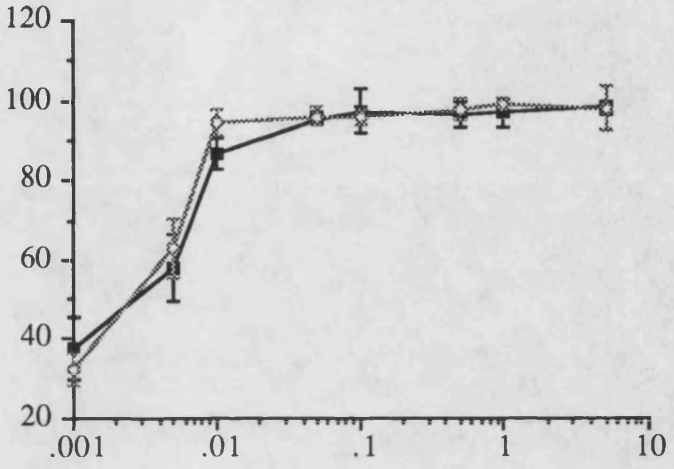


Figure 7.5 Effect of IL-1β on adhesion to ECM proteins.

MG-63 cells were cultured in MEM+3% CS-FCS +/- IL-1 β (10 U/ml). After 72 hr, cells were resuspended in serum free medium and added to microtitre wells coated with collagen I (A), fibronectin (B) and laminin (C). After 2 hr, % adhesion was assessed and values obtained at each ECM concentration from control and treated wells were compared using an unpaired Student's T-test. Points are means and standard deviations from three wells and are representative of three different experiments.
Basal adhesion to uncoated plastic = 22%

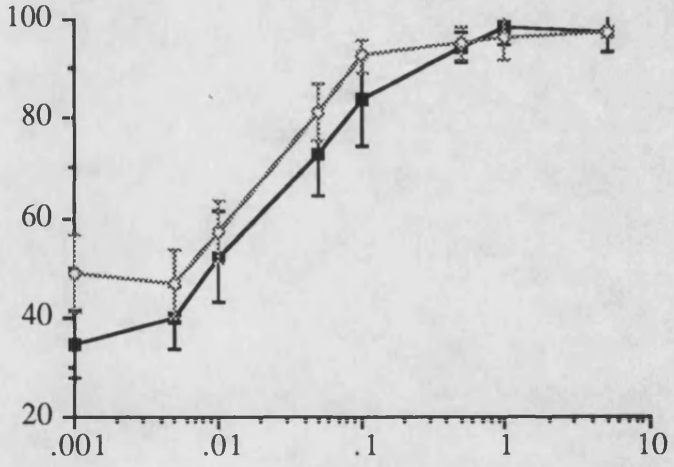
Figure 7.6

A. Collagen I



% Adhesion

B. Fibronectin



Control
TGFβ

cont...

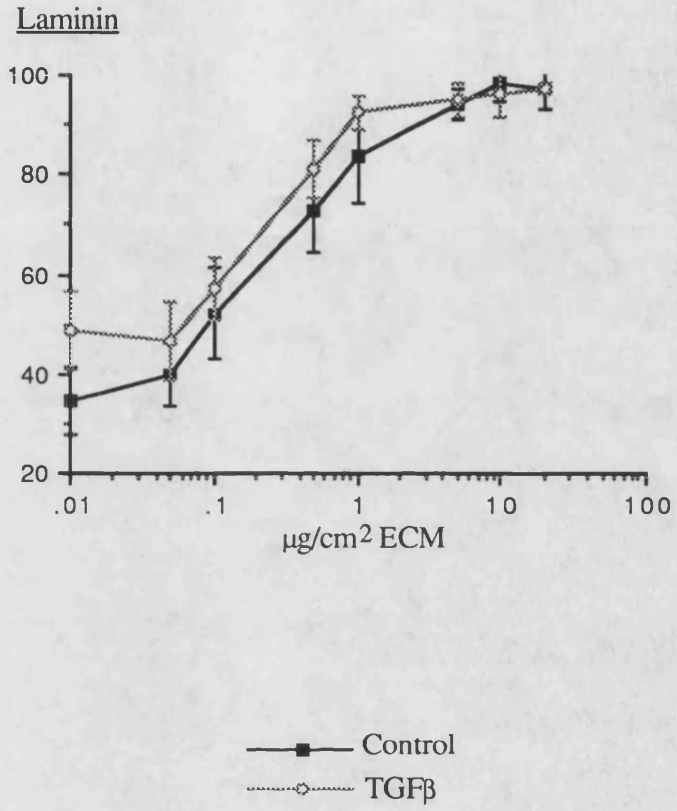


Figure 7.6 Effect of TGFβ on adhesion to ECM proteins.

MG-63 cells were cultured in MEM+3% CS-FCS +/- TGF β (25 ng/ml). After 72 hr, cells were resuspended in serum free medium and added to microtitre wells coated with collagen I (A), fibronectin (B) and laminin (C). After 2 hr, % adhesion was assessed and values obtained at each ECM concentration from control and treated wells were compared using an unpaired Student's T-test. Points are means and standard deviations from three wells and are representative of three different experiments.
Basal adhesion to uncoated plastic = 38%

CHAPTER 8.

FINAL DISCUSSION

The aim of this project was to investigate the role of cell-matrix interactions in the regulation of human bone cell function. Growth of human osteoblast-like cells on fibronectin, laminin, collagen I film and collagen I gel exerted a variety of effects on proliferation, alkaline phosphatase activity and 1,25D induced osteocalcin release (Chapter 3). However, none of these effects could be correlated with quantitative changes in integrin subunit expression (Chapter 5). It is possible that growth on different matrix components promoted a re-distribution of integrin subunits. In support of this, it has been demonstrated that the organisation of integrin receptors in fibroblasts and human endothelial cells is specifically determined by ECM molecules (Dejana et al.1988; Singer et al.1988). Alternatively, different matrix molecules could affect the internal tension of the cytoskeleton through their specific shear strengths (Bill Whish; personal communication). These phenomena would not necessarily affect the number of integrin subunits expressed but they would alter their cytoskeletal associations. Bissell and Barcellos-Hoff (1987) have suggested that the organisation of the cytoskeleton, which is determined by the ECM, is critical for expression of a particular cell phenotype. These workers found that primary cultures of mouse mammary epithelial cells expressed a more differentiated phenotype (as indicated by increased expression of β-casein and transferrin mRNA) when cultured on 'released' collagen I gel or matrix from Engelbreth-Holmes-Swarm tumour (EHS). They postulated that this relationship results from an increase in mRNA half life occurring both in the nucleus, as a result of cytoskeleton-nuclear matrix interactions, and in the cytoplasm as the result of polysome-cytoskeleton interactions.

The only matrix-mediated cellular response that could be correlated directly with a change in the cell surface expression of integrin subunits occurred following growth on collagen I gel. This matrix increased expression of α2 integrin subunits by 3.5 fold and was directly associated with a change in cell morphology and gel contraction. The mechanism of gel contraction is thought to involve changes in the cytoskeleton as it does not involve the breakdown of collagen and is prevented following the addition of colchicine (Strom and Michalopoulos, 1982). Gel contraction probably results from a difference in internal and external forces and supports the hypothesis that interactions between the ECM and the cytoskeleton play an important role in regulating cell function. This finding also demonstrates that different integrin molecules can translate specific signals into events which physically modify the structure of the matrix. The ability of a cell to organise its collagen matrix is essential for the formation of lamellar bone where collagen fibres are arranged in a distinct orientation and mineralisation occurs at specific foci. These characteristics contribute to the compressive strength and

rigidity of lamellar bone and are in sharp contrast with those of the mechanically inefficient woven bone where collagen fibres appear as disorganised bundles.

Integrin α subunits were differentially expressed by cells of the osteoblastic lineage *in situ* (Chapter 4) and were the major integrin subunits to be regulated by cytokines and ECM components *in vitro* (Chapters 5 and 6). *In situ* hybridisation studies performed by other members of our laboratory have localised expression of IL-1β and TGFβ to specific areas of active bone formation. Taken together, these findings could suggest that modulation of integrin subunit expression in response to transient expression of IL-1β and TGFβ is important during the formation phase of the bone remodelling cycle. We were unable to correlate a modulation of integrin subunit expression with changes in the differentiated osteoblast phenotype (Chapter 5) or with cell adhesion (Chapter 7). However, integrin molecules regulate many diverse aspects of cell phenotype, including gene induction (Werb et al.1989; Dhawan and Farmer, 1990; Dhawan et al.1991; Seftor et al.1992), phagocytosis (Graham et al.1989; Savill et al.1990) and migration (Chan et al.1992). It is therefore possible that regulation of integrin subunit expression by cytokines is associated with other phenotypic effects which have yet to be established.

In addition to IL-1β and TGFβ, there are many other soluble mediators which affect the actions of osteoblasts and osteoclasts and regulate integrin subunit expression (see Introduction). Furthermore, many of the factors considered to play a role in the process of bone remodelling including IL-1β, TGFβ, IGF-I and II and 1,25D also stimulate ECM synthesis (see Introduction). ECM molecules influence the presentation of cytokines and growth factors (see Introduction) and in addition modulate integrin subunit expression (Chapter 5). ECM components have also been demonstrated to increase cytokine release (Nathan and Sporn, 1991). For example, IL-1 release from human peripheral blood monocytes is stimulated by collagen fragments, hydroxyapatite and to a lesser extent by TGFβ (Pacifici et al.1991). These interactions between soluble mediators, ECM components and integrin subunit expression could provide some of the links required to increase our understanding of the regulatory mechanisms controlling the bone remodelling cycle.

The modulation of integrin subunit expression which occurred in response to cytokines and ECM components provides further evidence to suggest that integrin molecules are performing some kind of signalling role. Changes in cytoskeletal associations and other biochemical signals could occur through changes in tyrosine phosphorylation. In support of this, it has been shown that clustering of β1 integrins, a process which is

observed during the formation of adhesive contacts between cells and their ECM, is specifically associated with enhanced tyrosine phosphorylation of a complex of proteins of 115-130 KD (Kornberg et al.1991). It has also been demonstrated that adhesion of mouse fibroblasts to substrata coated with fibronectin causes increased tyrosine phosphorylation of proteins of about 120 KD, whereas adhesion of cells to substrata coated with non-specific ligands such as polylysine failed to produce this effect (Guan et al.1991). One of the components of this 115-130 KD tyrosine-phosphorylated complex, a 125 KD protein, encodes a novel tyrosine kinase from the src family and is termed pp125 Focal Adhesion Kinase ($pp125^{fak}$) (Hanks et al.1992; Schaller et al.1992). Observations in platelets also suggest a role for integrins in tyrosine kinase-mediated signal transduction. When platelets are activated by agonists, the $\alpha IIb\beta 3$ receptor complex undergoes a conformational change which exposes a binding site for fibrinogen (Shattil et al.1985). This results in receptor clustering, a subsequent association of receptor complexes with cytoskeletal proteins (Phillips et al.1980) and a stimulation of tyrosine kinase activity which occurs in three temporal waves (Tusznski, 1987; Ferrell and Martin, 1989; Golden et al.1990). Recently a strong correlation has been demonstrated between ligation of platelet integrins and activation of $pp125^{fak}$ (Lipfert et al.1992). These examples provide compelling evidence to suggest that ligation of integrins, followed by receptor clustering and cytoskeletal reorganisation, can affect $pp125^{fak}$, leading to activation and increased tyrosine phosphorylation of this protein. Therefore it seems likely that integrin-mediated activation of $pp125^{fak}$ is an early step in the signal transduction cascade which permits the flow of information between the external environment and the interior of the cell.

Signal transduction via the integrins does not necessarily require a change in the level of cell surface expression. This could be important in the case of the osteoclast in which there is constitutive expression of integrin subunits *in situ*, irrespective of their location in bone (Chapter 4). Evidence is emerging that signal transduction in osteoclasts involves the *src* gene which encodes a $pp60^{c\text{-}src}$ tyrosine kinase. c-*src* is critical for osteoclast activity since targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice (Soriano et al.1991): three other *src*-like kinases c-*fyn*, c-*yes* and c-*lyn* are expressed at normal levels in osteoclasts from *src* deficient mice but these proteins do not compensate for the absence of $pp60^{c\text{-}src}$ (Horne et al.1992). $pp60^{c\text{-}src}$ is highly expressed on the ruffled border of osteoclasts (Tanaka et al.1992) and is required for the formation of these membranes (Boyce et al.1992). Furthermore, binding of a cyclic RGD peptide to isolated osteoclasts induces a wave of tyrosine phosphorylation and the translocation of a *src* substrate p85 (Neff et al.1992). In another study, occupancy of the osteoclast $\alpha V\beta 3$ by osteopontin stimulates a *src*

associated phosphatidylinositol 3 kinase (PI3 kinase) resulting in phosphatidylinositol trisphosphate (PIP_3) formation (Hruska et al.1992). The *src* substrate p85 is one of the two subunits comprising PI3 kinase so taken together, these findings provide compelling evidence to suggest that integrin-mediated tyrosine phosphorylation may be an important mechanism of signal transduction in osteoclasts.

In addition to changes in phosphorylation, a number of other integrin-mediated signalling events have been described. For example, in platelets there is evidence that integrin ligation can affect calcium activated proteases, the Na^+/H^+ antiporter and the subcellular distribution of PI3 kinase as well as influencing patterns of tyrosine phosphorylation. Osteopontin can influence calcium transients in osteoclasts via the $\alpha V\beta 3$ integrin (Miyauchi et al.1991), while both cAMP and calcium are affected by $\beta 2$ integrin-mediated events in neutrophils. In addition, signalling events involving arachidonic acid metabolites have been associated with integrin-dependent cell adhesion. The relationship of these additional processes to integrin-mediated tyrosine phosphorylation is at present unknown (reviewed by Juliano and Haskill, 1993).

The ability of integrins to function as true signalling receptors suggests that the activities of integrin molecules can be regulated by both extracellular and intracellular signals to elicit a specific cellular response. This could have important implications in the context of bone remodelling and it helps to explain why binding of integrin molecules to ECM components modulates cell activities in ways which extend far beyond adhesion. For example, it could account for gel contraction and the changes in differentiated osteoblast phenotype observed *in vitro* during experimental studies. One could also speculate that integrin-mediated signals elicited by the ECM could affect the development and differentiation state of osteoblasts *in vivo* thus influencing matrix deposition and the formation of osteoblast lining cells and osteocytes. An important area which warrants further investigation concerns the role of integrin-mediated signals elicited in response to mechanical strain. Profound changes in bone modelling and remodelling occur after only very short periods of loading (Rubin and Lanyon, 1985). Evidence suggests that this involves increased biosynthetic activity of osteocytes (Skerry et al.1989) and changes in the orientation of proteoglycans within the bone matrix (Skerry et al.1990). Although osteocytes themselves are not thought capable of bone formation, they could translate signals to osteoblasts through gap junctions or through local cytokine production. Since integrins form a direct link between the ECM and the cytoskeleton, these cell surface receptors could potentially initiate the osteocytic response. Induction of bone resorption would not necessarily require signals from osteocytes. Osteoblast lining cells are also appropriately positioned to detect changes in

mechanical strain (Paed et al.1988). One could speculate that changes in mechanical loading could be transmitted to the interior of the osteoblast lining cell via the integrins. This could induce cytokine release which in turn could initiate bone resorption and subsequent bone formation. This process would continue until the increased strain was no longer detected by cells of the osteoblastic lineage and the new structure was optimally suited to the prevailing mechanical load.

Integrin-mediated signalling could also be of vital importance to the osteoclast. For example one could postulate that initiation of bone resorption, which requires attachment of mononuclear precursor cells to the bone matrix involves integrin-mediated activation. Similarly, the formation of multinucleated osteoclasts, which requires fusion of mononuclear cells, and the process of bone resorption, which requires an intimate association between the sealing zone of the osteoclast and its ECM, could also be associated with integrin-mediated activation. Of equal importance with activation of integrins is their inactivation: this could be important during the reversal stage of the bone remodelling cycle when osteoclasts detach from the bone matrix and perhaps undergo fission to form TRAP positive mononuclear cells.

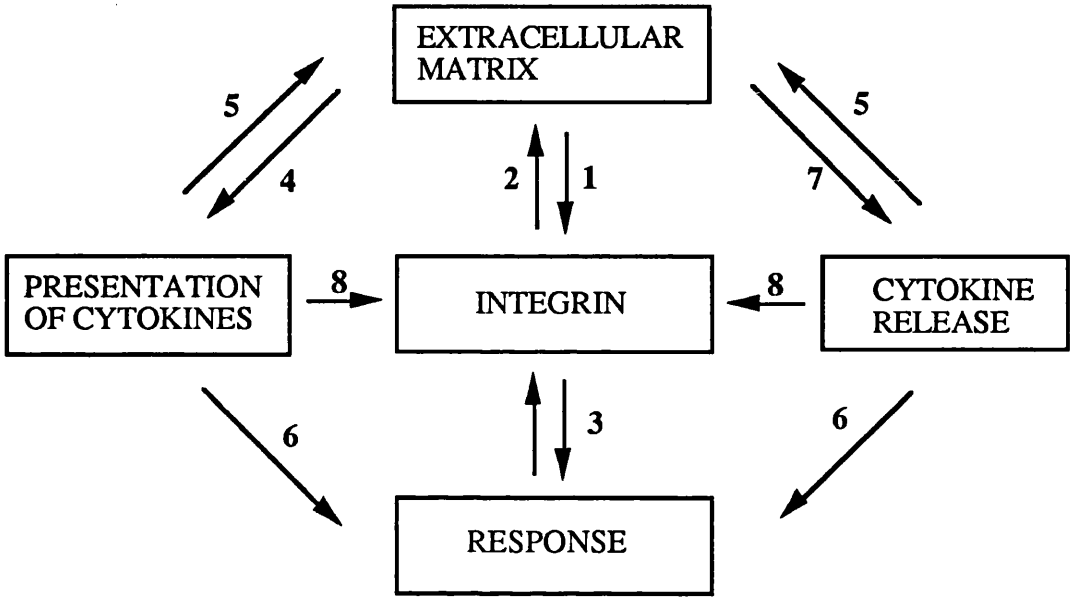
Most of these examples rely on integrin molecules responding to external stimuli elicited by the ECM. However, it is equally possible that signals transmitted from within the cell affect integrin activation. This phenomenon is perhaps best illustrated in non-adherent cells such as lymphocytes. Stimulation of the T-cell antigen receptor/CD3 complex augments the affinity of certain integrins for ligand, promoting transient cell adhesion (Springer, 1990). This could be of importance in the case of the osteoclast during the reversal phase of the bone remodelling cycle with detachment from the bone surface (and osteoclast fission?) possibly resulting from an intracellular inactivation of integrin receptors.

The biochemical signals activated by integrin receptor clustering are similar to those elicited by several growth factor receptors following ligand binding. This suggests that signalling pathways triggered by soluble growth factors and ECM molecules could converge and/or synergise with each other to regulate cell growth and differentiation. In fact integrins could be required in conjunction with growth factor receptors to regulate cell phenotype. This type of relationship can be observed using mouse mammary epithelial cells. Terminal differentiation of alveolar structures and production of milk proteins require both lactogenic hormones and contact with the basement membrane. The signal from the basement membrane is integrin mediated, as antibodies against the β1 integrin family block β-casein production (Streuli et al.1991). A 161

base pair enhancer in the 5' regulatory region of the β-casein gene conveys responsiveness to ECM and lactogenic hormones. This enhancer does not exhibit any sequence homology with other nuclear protein-binding sites, suggesting that specific matrix-response elements are required for expression of the correct cell phenotype (Schmidhauser et al.1992). Therefore, in addition to providing temporal and spatial localisation of cytokines and growth factors, ECM components may be required or could at least amplify a cellular response. This means that soluble and insoluble factors cannot be considered in isolation and that cell-matrix interactions play a vital role in the control of a given cell type. The potential interplay between ECM, integrins and cytokines in the local control of bone remodelling is summarised in Figure 8.1.

There are many unanswered questions concerning bone remodelling and the scheme depicted in Figure 8.1 only represents a fraction of the overall potential regulatory network. Hopefully as research progresses it will be possible to unravel more of these regulatory mechanisms, and the underlying causes of bone diseases can be addressed.

Figure 8.1



A schematic representation of the potential interplay between ECM, integrins and cytokines during bone remodelling.

1. ECM molecules determine the distribution of integrin receptors and modulate their cell surface expression.

2. By binding to different ligands, the integrin subunit expression determines the microenvironment immediately surrounding the cell.

3. Two way signalling between the ECM and interior of the cell is mediated via the integrins through activation of biochemical secondary messengers and/or changes in cytoskeletal associations.

4. Matrix molecules present cytokines and growth factors to neighbouring cells.

5. Cytokines affect matrix production.

6. Cytokines elicit a cellular response.

Signals elicited by 3 and 6 could converge and synergise with each other to amplify a cellular response.

7. Matrix molecules affect cytokine release.

8. Cytokines modulate integrin receptor function.

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